CALCIUM-ACTIVATED POTASSIUM-CHANNELS IN MAMMALIAN EGGS

by

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THESIS

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I dedicate this thesis to my beloved "mother" Panayiota and to my late godfather Demetrios whose great sacrifices enabled me to have my education and to whom I will be always extremely grateful.

DECLARATION

I certify that the work presented in this thesis is my own.

P. GEORGIOU.

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CHAPTER III

THE EFFECT OF MULTIVALENT IONS AND DINITROPHENOL (DNP) ON THE CALCIUM-EVOKED HYPERPOLARIZATION

SUMMARY

Intracellular microelectrode recordings from zona-free hamster eggs revealed a mean value for the membrane potential of -34±9mV (mean ± SD, n=36) and a mean value for the input resistance of 290±170MΩ(mean ± SD).

Ionophoretic injection of calcium ions into a hamster egg caused a marked membrane hyperpolarization (mean ± SD = 27±9mV, n=63) which was associated with a large reduction in the egg input resistance (viz. from 140±94MΩ prior to the injection to 40±24MΩat the peak of the response, n=63). The estimated reversal potential of the calcium-evoked hyperpolarization was found to be -69±11mV (mean ± SD,n=63). The mean slope of the relation between the reversal potential and $\log_{10}[K^+]_o$ was 50mV in good agreement with the value predicted by the Nernst equation for a rise in conductance primarily selective for potassium ions. In addition the reversal potential was unaffected by about a tenfold reduction in the concentration of external chloride. It is concluded that the calcium-evoked hyperpolarization was caused by the opening of membrane potassium channels that are activated by an increase in the intracellular concentration of ionized free calcium.

The duration of the calcium-evoked response was markedly extended in the presence of 1mM external lanthanum or 20mM calcium or by external application of 0.2-2mM 2,4,dinitrophenol.

Double microelectrode experiments identified the presence of an impalement leak artifact which might cause an underestimate of the true membrane potential and input resistance. Evidence is also presented for a second type of artifact, namely the generation of a leak conductance pathway during the passage of large depolarizing current pulses (>10nA, 1 sec) used for the ionophoretic injection of calcium into cells.

INTRODUCTION

It is now generally accepted that the lipid component of cell membranes is impermeable to ion flow, and that ions are able to cross cellular membranes via protein generated channels or pores. In many instances channels exhibit strong selectivity towards the nature of the traversing ion, acting as molecular sieves by imposing limits to ion valence and size.

Certain types of channels fall under the general category of "voltage-sensitive". As the name implies the ability of these channels to conduct ionic current depends exclusively on the difference in transmembrane voltage.

Channels are also found under the general category of "ligand-operated" whose gating or conductance characteristics depend primarily on the concentration of specific molecules or ions, referred to as ligands, present either on the extracellular or the intracellular membrane side. The binding of these specific ligands to the complementary channel receptor site(s) alters the channel configuration from a non-conducting to a conducting one. Membrane voltage can modulate the function of these channels by changing, for example, the rate at which the ligand-receptor binding takes place.

The subject of this thesis is mainly concerned with a certain type of ligand-operated channel, namely the Ca^{2+}-activated K^{+}-channel. This channel is

primarily selective for potassium ions (K^+) and opens in response to a rise in the intracellular concentration of free calcium ions (Ca^{2+}). It is the purpose of the rest of this introduction to consider aspects of what is currently known about Ca^{2+}-activated K^+-channels, based largely on results obtained from electrophysiological investigations.

Section 1 :The Gardos Effect

Early studies on red cells (Henriques & Orskov, 1936; Wilbrandt, 1937, 1940 ; reviewed by Lew & Ferreira, 1978 and by Schwarz & Passow, 1983) established that treatment of red cells with metabolic inhibitors such as iodoacetate or fluoride induced a selective rise in the K^+ efflux of the red cell membrane. Gardos (1958) found that the net K^+ efflux failed to occur in the absence of external calcium. Credit, however, for the concept of a Ca^{2+}-mediated increase in the membrane permeability to K^+ goes to Whittam who first proposed it in 1968. Lew (1970) presented evidence that suggested the existence of a close relationship between variation in the concentration of intracellular free calcium and alterations in the K^+ permeability of the red cell membrane.

Section 2 :The presence of Ca^{2+}-activated K^+-channels in a variety of cells and tissues

Since the discovery of Ca^{2+}-activated K^+-channels in red cells numerous other cells and tissues have been reported to possess them. Examples of cells and tissues where the presence of Ca^{2+}-activated K^+ channels has been established by electrophysiological investigations are given in Table 1. An asterisk under the column labelled preparation means that indirect evidence exists for the presence of Ca^{2+}-activated K^+-channels in that particular cell or tissue, although direct verification has not yet been proved possible .

Table 1 Ca^{2+}-activated K^+ channels found in different preparations.

Preparation	Techniques used to demonstrate the presence of Ca^{2+}-activated K^+ channels and the results obtained.	References
Molluscan Neurones		
Helix aspera identified neurones of the suboesophageal ganglion Aplysia californica	Intracellular microelectrode recordings from Aplysia californica or Helix aspera neurones have shown that the immediate effect of intracellular calcium injections was a membrane hyperpolarization associated with a marked reduction in cell input resistance. The reversal potential of the response shifted by 58mV for a tenfold change in $[K^+]_o$. Repetitive depolarizing current pulses applied through the recording microelectrode could also elicit a hyperpolarization which was again accompanied by a substantial reduction in cell input resistance. Both application of 1mM 2,4 dinitrophenol and 2mM iodoacetate greatly potentiated the reduction in input resistance and the duration of the response was greatly prolonged.	Meech & Strumwasser (1970) Meech (1974a)
Helix aspera Identified neurone (cell A) of the suboesophageal ganglion	Demonstration by voltage clamp experiments of an outward current elicited in response to depolarizing command pulses. Bathing the preparation in a calcium-free solution resulted in diminution of the observed outward current. Double step potential analysis of tail currents identified two exponentially declining components, one of which could be suppressed in calcium-free solution.	Meech & Standen (1975)

Molluscan Neurones

Aplysia californica R_2 giant neurone of the abdominal ganglion	The light activated potassium current of the giant neuron Aplysia californica, monitored under voltage clamp, was enhanced by injection of Ca^{2+}-EGTA buffers adjusted to give an effective free calcium concentration of 0.28-0.56μM. Injection of Ca^{2+}-EGTA buffers adjusted to give a free calcium concentration of 0.056μM greatly reduced the light activated outward current. TEA suppressed both light and calcium evoked outward currents.	Brown, Brodwick & Eaton (1977)
Anisodoris nobilis giant neurones	The calcium sensitive photoprotein aqueorin, in conjunction with voltage clamp, were used to show that during membrane depolarization a close correlation existed between a rise in cytosolic free calcium concentration effected by inward calcium movement through voltage gated calcium channels and the development of an outward potassium current.	Eckert & Tillotson (1978)
Neurones of Helix pomatia Right parietal ganglion	Single channel currents recorded using the patch clamp recording method in the cell-attached configuration were identified as K^+ channels activated by Ca^{2+} on the basis that all time-dependent currents elicited by depolarization were reduced by more than 80% in calcium free bathing solutions. In patches where the pipette solution in contact with the external membrane surface contained 40mM K^+ instead of the normal 4mM, the amplitude-voltage relationship shifted by about 50mM, as would be expected for a channel primarily selective for K^+.	Lux, Neher & Marty (1981)
Aplysia californica Pacemaker neurones (R-15) neurone of the abdominal ganglion	Intracellular ionophoretic injection of Ca^{2+} into R-15 neurones held under voltage clamp elicited an outward K^+ current with a selectivity sequence of $Tl^+ \leqslant K^+ > Rb^+ > NH_4^+ > Cs^+ > Na^+, Li$.	Gorman, Woolum & Cornwall (1982)

Other tissues

Cell culture of rat skeletal muscle	Application of the patch clamp recording technique to show that in excised inside out membrane patches an increase in the free calcium concentration on the intracellular membrane side evoked opening of channels carrying outward currents. Alteration of the K^+ gradient across the patch produced a shift in the reversal potential of the channel in agreement with that expected for a K^+ selective electrode. Total replacement of Cl^- with SO_4^{2-} or 2(N-morpholino) ethane sulphuric acid (MES) had no effect on either the reversal potential or the single channel conductance. The latter had a value of 187pS when identical K^+ solutions were applied on both sides of the membrane.	Pallotta, Magleby & Barret (1981)
Cultured rat myoballs	Use of the patch clamp technique in the outside-out configuration revealed the presence of channels activated by internal calcium, since both open channel lifetimes and frequency of opening were progressively enhanced by a rise in free calcium from 0.001 to 0.005 and then to 1μM. The channel had a reversal potential of -65mV when the external solution bathing the patch was 150mM NaCl and the internal solution was 150mM KCl. The selectivity ratio $P_{Na}/P_K = 0.07$ as determined from reversal potential measurements. The single channel conductance varied from 180-260 pS in symmetrical K^+ solutions (150mM KCl on both sides of the membrane).	Methfessel & Boheim (1982)

Bullfrog sympathetic ganglia	Conventional intracellular recordings from bullfrog sympathetic neurones in the presence of caffeine have shown that the membrane potential of these neurones undergoes repetitive hyperpolarizations. Ionic substitution experiments revealed that the caffeine induced hyperpolarizations were dependent on the presence of $[K^+]_o$ but not $[Cl^-]_o$. Intracellular calcium injections can evoke hyperpolarizing responses similar to the ones produced by caffeine whereas injection of the calcium-chelating agent EGTA blocked the generation of caffeine induced hyperpolarizations	Kuba, Minota & Nishi (1972) Kuba & Nishi (1976) Kuba (1980)
Bullfrog lumbar sympathetic neurones	Intracellular calcium injections into voltage clamped neurones generated an outward current with a reversal potential of -35mV in 25mM $[K^+]_o$. Currents generated by Ca^{2+} injections were totally eliminated by both application of the tetraethylammonium ion (TEA), a K^+ channel blocker. Single channel currents recorded by patch clamping neurones in the cell-attached configuration have a single channel conductance of 100pS.	Adams, Constanti, Brown & Clark (1982)

Cells in the adenohyphysis of ovariectomised rats *	Intracellular recordings from this preparation identified a population of cells with a mean resting potential of -37 ± 2mV and input resistance of 111 ± 31MΩ which exhibited spontaneous, repetitive hyperpolarizations. These hyperpolarizations could be reversed to depolarizations by current clamping the membrane potential to values greater than their reversal potential, which had a value ranging from -55 to -70mV. A tenfold rise in $[K^+]_o$ produced about a 3-fold reduction in the amplitude of the observed hyperpolarizations.	**Poulsen & Williams (1976)**
Sheep cardiac Purkinje fibres *	Injection of Ca^{2+} into cardiac Purkinje fibres shortened the action potential, augmented the after-potential and caused the resting membrane potential to hyperpolarize. Intracellular EGTA injection lengthened the action potential and abolished the after-potential.	**Isenberg (1977)**

Guinea-pig hippocampal slices *	A depolarizing current pulse delivered via an intracellular microelectrode induced repetitive firing in hippocampal CAI neurones, which was followed by a long lasting after-hyperpolarization (AHP). During the AHP the membrane conductance rose by 10-25% whereas intracellular ionophoresis of Cl^- had no effect on the AHP extracellular perfusion whereas 0.2-μM Ba^{2+} eliminated it. The AHP was abolished by application of the calcium channel blocker Mn^{2+} but not by the sodium channel blocker tetrodotoxin (TTX) and appeared to be temporally associated with TTX resistant "Ca^{2+} action potentials".	Hotson & Prince (1980)
Guinea-pig brain stem slices *	Intracellular recording from inferior olivary neurones (I.O.) revealed that antidromic, orthodromic or direct stimulation initiated action potentials consisting of a fast spike followed by a plateau depolarization (after-depolarization potential). The after-depolarizing potential (ADP) was immediately followed by a prominent after-hyperpolarization potential (AHP) of 12mV and lasting up to 250m sec. Both application of tetrodotoxin (TTX) and removal of extracellular Na^+ eliminated the fast spike without affecting either the ADP or the AHP. However, bath application of any one of the following calcium channel blockers, namely Co^{2+}, Mn^{2+}, Cd^{2+} or D600, or substitution of Ca^{2+} by Mg^{2+}, abolished both the ADP and the AHP.	Llinas & Yarom (1981)

Mouse neuroblastoma cells *	A single microelectrode was used for both current passing and voltage recording. Action potentials with a substantial calcium component were observed in these cells. A prolonged after-hyperpolarization was seen to follow such spikes. Ba^{2+} or Sr^{2+} (20mM) could substitute for Ca^{2+} in producing action potentials (in Na^{+}-free solutions) but the after-hyperpolarization was abolished. Bath application of TEA (15mM) increased the peak value of the action potential, prolonged the repolarizing phase and enhanced the duration of the after-hyperpolarization. The after-hyperpolarization was suppressed by the Ca^{2+} channel antagonists La^{3+}, Co^{2+} and Mn^{2+}.	Moolenaar & Spector (1979)
	Voltage clamp experiments performed in solutions containing 20mM $[Ca^{2+}]_o$ and 25mM external (TEA) revealed the presence of a slow outward current upon prolonged depolarizations to values greater than -20mV. Slowly declining tail currents reversed their direction in a range of holding potentials lying between -70 to -85mV. An increase in the external $[K^{+}]_o$ from 5.5 to 55mM shifted the reversal potential to -25mV, a change consistent with the prediction of the Nernst equation for a conductance primarily selective for K^{+} ions. This slow K^{+} current was blocked by external Co^{2+} (1.8mM) and La^{3+} (2mM).	Moolenaar & Spector (1979)
Paramecium tetraurelia *	Intracellular Ca^{2+} injections into Paramecium tetraurelia produced a transient membrane hyperpolarization of about 5mV and a reduction in input resistance of about 25%. On the other hand intracellular EGTA injection caused a 100% rise in input resistance accompanied with a depolarization. In the presence of intracellular applied TEA, EGTA injection produced no further increase in potential or resistance. Based mainly on these observations Satow suggested that the resting potential in Paramecium tetraurelia is predominantly governed by a K^{+} conductance that is in turn controlled by the concentration of intracellular free calcium ions.	Satow (1977)

Clonal anterior pituitary cells	The patch clamp recording technique was employed both in the cell-attached and the inside-out configuration to record single channel activity under different intracellular free calcium concentrations. At an internal free calcium concentration of $\leq 0.01\mu M$ channel openings were rarely observed. Elevation of $[Ca^{2+}]_i$ from 0.01 to $0.1\mu M$ slightly increased the mean open time and the frequency of channel openings. A pronounced rise in open state probability and a marked increase in mean open time was observed when the $[Ca^{2+}]_i$ was raised from 0.1 to $1\mu M$. In normal and reversed physiological K^+ gradients the corresponding reversal potentials were -85 and +89mV respectively whereas under identical 145mM K^+ solutions across the patch the reversal potential was zero. The shift in the reversal potential was consistent with the interpretation that the channels are primarily selective for K^+.	**Wong, Lecar & Adler (1982)**
Mouse pancreatic β-cells	Intracellular microelectrode recordings from pancreatic β-cells have shown that bath application of quinine, a blocker of Ca^{2+}-activated K^+ channels (Armando-Hardy, Ellory, Ferreira, Fleminger & Lew,1975) eliminated the glucose-evoked regular bursts of cation potentials. Quinine also counteracted the hyperpolarization induced in pancreatic β-cells by a variety of mitochondrial inhibitors. The latter were known to liberate Ca^{2+} from intracellular stores (Rojas & Hidalgo, 1968; Blaustein & Godgkin, 1969; see also results obtained by Meech 1974, tabulated at the start of this table.) The authors proposed that the action of these inhibitors was to release Ca^{2+} from mitochondrial stores which in turn activated a Ca^{2+}-sensitive K^+ conductance. Quinine by blocking the K^+ conductance reduced or abolished the observed hyperpolarization.	**Atwater, Dawson, Ribalet & Rojas (1979)**

Rat pancreatic islet cells *	Identification, through single channel recordings from excised inside-out membrane patches, of channels whose open state probability increased considerably, at a fixed membrane voltage, following a rise in the intracellular concentration of free calcium ions. These channels had a conductance of 240pS when both sides of the membrane patch were in contact with identical K^+ solutions (140mM). Cook et al (1980) have tentatively identified them as Ca^{2+}-activated K^+ channels, presumably from their large single channel conductance, which closely resembled the single channel conductance of identified Ca^{2+}-activated K^+ channels. Experiments to determine their selectivity towards K^+ have not been carried out.	Cook, Ikeuchi & Fujimoto (1984)

Pig pancreatic acinar cells	Again a combination of patch clamp recording technique and excised membrane patches demonstrated the existence of outward channels activated by 0.1μM internal calcium. Reversal of outward currents was not observed but the reversal potential was more negative than -40mV in the absence of Cl^- gradient, therefore outward currents must be caused by outward movement of K^+.	**Maruyama, Petersen, Flanagan & Pearson (1983)**
Cockroach (Periplaneta americana) central neurones viz cell 28 of the metathoracic ganglion	Voltage clamp analysis revealed an outward current which was suppressed in Ca-free saline. Intracellular calcium injections elicited an outward current whose reversal potential varied with $[K^+]_o$ as would be expected from the Nernst equation.	**Thomas (1984)**
Smooth muscle cells obtained from the stomach of the toad Bufo marinus (by enzymic digestion)	Microelectrode insertion into these cells produced a transient hyperpolarization with a concomitant reduction in input resistance. Patch clamp studies of cell-free membrane patches show that calcium at μM concentrations evoked outward currents whose reversal potential varied with the K^+ concentration across the patch. The single channel conductance was 250pS in symmetrical (130mM) $[K^+]$ across the patch.	**Walsh & Singer (1983)**
Acinar cells of the mouse exorbital lacrimal gland	Intracellular microionophoresis of Ca^{2+} into surface acinar cells caused membrane hyperpolarization and a reduction in cell input resistance. The value of reversal potential calculated from the potential and resistance measurements was 150mV. In lacrimal acinar cells Ca^{2+} injections mimicked the action of acetylcholine or adrenaline.	**Iwatsuki & Petersen (1978)**

Cultured bovine adrenal chromaffin cells	Patch clamp recordings from cell-attached patches revealed the presence of large single channel currents which were found to be K^+ selective, as indicated by the fact that the reversal potential changed from a value of -60mV at $[K^+]_o$ = 2.8mM to a value of omV at $[K^+]$ = 143mM. Recording from inside-out patches under symmetrical K^+ solutions ($[K^+] \fallingdotseq$ 143mM on both sides of the membrane), showed that calcium applied at a concentration of 0.001 to 0.002 μM on the intracellular side caused channel opening when the membrane potential was displaced from 0 to +67mV. At a $[Ca^{2+}]_i$ = 0.001μM the average number of open channels at the end of the positive step was 0.51 whereas at $[Ca^{2+}]_i$ = 0.02μM it increased to 1.7, indicating that calcium increased the average number of open channels. The amplitude of the single channel current was lower at the higher calcium concentration. When $[Ca^{2+}]_i$ = 1mM, the single channel conductance decreased, the mean number of channels increased and the voltage dependence was lost. The single channel conductance was found to be 180pS in symmetrical K^+ solutions.	Marty (1981)

Cultured human macrophages	Single channel currents recorded in intact and excised membrane patches through the use of the patch clamp recording method were found to respond to a rise in the concentration of ionized free calcium by increasing their frequency of opening and their mean open time. In excised membrane patches the reversal potential changed in accordance with the electrochemical K^+ gradient across the patch, thus indicating that the channel is primarily selective for K^+. Single channel conductance varied with the K^+ gradient across the patch, being highest (240pS) when both sides of the membrane were bathed in identical (145mM) K^+ solutions.	Gallin (1984)
Cultured fibroblastic L-cells	Hyperpolarizing current pulses (1-50nA, 100 msec duration) delivered through an intracellular microelectrode, designed to record simultaneously changes in potential and resistance, elicited membrane hyperpolarizations lasting about 10sec. The peak of the hyperpolarizing response was associated with a marked reduction in input resistance. Mechanical displacement of the recording microelectrode or pressure exerted on the cell membrane by a second micropipette was also effective in triggering hyperpolarizations. Spontaneous recurring hyperpolarizations were also observed following microelectrode insertion into a cell. The reversal potential of the hyperpolarization was found to change by 37mV in response to a fivefold rise in the external K^+ concentration, thus suggesting that the hyperpolarization was caused primarily or exclusively by a preferential increase in the membrane conductance towards K^+ ions. Intracellular microionophoresis of Ca^{2+} while monitoring changes in potential and resistance by a second K-acetate microelectrode evoked a hyperpolarizing response the amplitude of which was proportional to the strength of the Ca^{2+} current pulse.	Nelson, Peacock & Minna (1972)

Hamster eggs	Fertilization of hamster eggs in vitro was found to be associated with recurring membrane hyperpolarizations as revealed by intracellular microelectrode recordings. The reversal potential of the sperm-evoked responses shifted by 35.9mV for a fourfold increase in $[K^+]_o$. The expected shift according to the Nernst equation is 36.3mV, which suggests that the sperm-evoked hyperpolarizations are generated almost exclusively by a rise in the conductance to K^+. Following intracellular EGTA injections sperm-evoked responses were abolished whereas intracellular Ca^{2+} injections produced hyperpolarizing responses with a reversal potential of -80 to -85mV. The Ca^{2+}-evoked hyperpolarizations were accompanied by a fourfold increase in the K^+ conductance (calculated by substraction of the membrane conductance just before injection from that at the peak of the hyperpolarization). Removal of external calcium abolished sperm-evoked hyperpolarizations. The frequency at which sperm-evoked hyperpolarizations were repeated was reduced by lowering $[Ca^{2+}]_o$ or by adding Mv^{2+} or Co^{2+} to the solution bathing the eggs. The conclusion reached by Miyazaki and Igusa was that sperm-evoked hyperpolarizations were caused by a rise in $[Ca^{2+}]_i$ probably as a result of Ca^{2+}-induced Ca^{2+} release from intracellular stores.	Miyazaki & Igusa (1981,1982) Igusa & Miyazaki (1983)

Incorporation of membrane vesicles containing Ca^{2+}-activated K^+ channels isolated from traverse tubule membranes of rabbit skeletal muscle	Single channel fluctuations and conductance measurements were performed by a two-electrode voltage clamp. The conductance of vesicles containing many channels was found to depend on the cis (side to which vesicles were added that corresponded to the cytoplasmic side in vivo) concentration of ionized free calcium. At a concentration of free calcium of 1mM the macroscopic K^+ conductance increased with depolarization. The open state probability of current fluctuations due to a single channel was also found to depend on both calcium and voltage. An increase in the free calcium concentration at any fixed voltage caused a translation of the open-state probability versus single channel conductance of 226pS in 0.1M K^+ solutions bathing both membrane surface.	Lattore, Vergara & Hidalgo (1982)
Incorporation of membrane vesicles from rat brain into planar lipid bilayers.	Free calcium on the cis side was required for the generation of single channels. EGTA addition to cis side abolished single channel currents. In assymetric solutions the reversal potential of single channel currents approximated the K^+ equilibrium potential. In 100mM symmetrical K^+ solutions the single channel conductance was 100pS.	Krulger, Blaustein, Worley & French (1982)

Section 3 :The opening of Ca^{2+}-activated K^+-channels is under the dual control of $[Ca^{2+}]$ and membrane potential

Meech and Strumwasser (1970) first showed that intracellular injection of calcium ions into Aplysia nerve cells caused a rise in K^+ conductance. Since then, their results have been confirmed and extended to numerous other preparations (see Table 1). It was not, however, realized until 1980, when Gorman and Thomas published their observations on the Ca^{2+}-activated K^+ conductance of Aplysia neurones, that the degree of activation of the Ca^{2+}-mediated K^+ conductance is also sensitive to alterations in membrane voltage. Gorman and Thomas (1980) first demonstrated that the Ca^{2+}-activated K^+ conductance increases e-fold in response to a 25mV change in membrane potential, provided the intracellular free calcium concentration is kept close to the minimum necessary for activation of the K^+-channels (just under 1.6μM).

With the advent of the patch clamp recording technique (Neher, Sakmann & Steinbach, 1978) a number of studies at the single channel level (see Table 1) have revealed directly that micromolar application of calcium ions at the inner side of the cell membrane promotes opening of K^+-channels, by increasing their frequency of opening (i.e. the number of times a channel opens per second) and their mean open time. Furthermore, in accordance with the observations of Gorman and Thomas (1980) the probability, p(V), of

finding a channel in the open state has also been shown be a function of the prevailing membrane voltage (Barret, Magleby & Pallota ,1982). Thus at any given voltage V, the open state probability, p(V), is described by a sigmoidal curve of the form:

$$p(V) = [1+ \exp(-zF(V-Vo))]^{-1} \quad .....(1)$$

where V_o is the voltage at which half of all the channels are open; z is the effective charge on the gating particle; F is the Faraday constant; R is the Gas constant and T is the absolute temperature (Kelvin Scale).

The voltage dependence of the Ca^{2+}-activated K^+ conductance or of the open state probability (when referring to a single channel) varies considerably among different preparations. This is illustrated in Table 2. Whenever possible experimental parameters such as pH_i and temperature have also been stated since they have been reported to influence the open-state probability of Ca^{2+}-activated K^+-channels (Cook, Ikeuchi & Fujimoto, 1984; Barret, Magleby & Pallotta, 1982).

The observed variability in the voltage dependence of Ca^{2+}-activated K^+-channels cannot be solely attributed to the experimental procedures used for its determination since in similar preparations approximately the same voltage dependence has been obtained by both the voltage clamp and the patch clamp recording methods. The reported differences in the voltage dependence of the Ca^{2+}-activated K^+-channels

Table 2 The voltage dependence of the Ca^{2+}-activated K^+ channel conductance in different preparations.

Preparation	Method used to obtain voltage dependence	Change in membrane potential needed to evoke an e-fold change in K^+ conductance or open state probability (mV)	$[Ca^{2+}]$ (μM)	pH_i	Temperature ^oC	Reference(s)
Aplysia californica R-15 neurone of the abdominal ganglion	Two electrode voltage clamp. Outward K^+ current generated by depolarizing command pulses	25	-	-	16	Gorman & Thomas (1980)
Helix aspera F cluster neurones	Patch clamp recordings from excised membrane patches in the inside-out configuration.	20.7 23.6	0.1 0.01	7.4 7.4	37 37	Ewald, Williams & Levitan (1985)
Periplaneta americana motorneuron cell bodies in the metathoracic ganglion	Two electrode voltage clamp. Outward K^+ current generated by depolarizing command pulses	28-38	-	-	20	Thomas (1984)

Bullfrog lumbar sympathetic neurons	Two electrode voltage clamp to monitor current flow across membrane. K^+ current evoked by intracellular Ca^{2+}-injections	11	-	-	22	Adams, Constanti, Brown & Clark (1982)
Clonal anterior pituitary cells	Patch clamp recordings from excised membrane patches in the inside-out configuration.	28	0.1	7.3-7.4	23-25	Wong, Lecar & Adler (1982)
Cultured rat skeletal muscle cells	same as above	15				Barrett, Magleby & Pallotta (1982)
Cultured rat myoballs	Patch clamp recordings from excised membrane patches in the outside-out configuration	12.6 12.6 12.4	1 2 50	- 7.2 -	- 18-20 -	Methfessel & Boheim (1982)
Ca^{2+}-activated K^+ channels from tranverse tubule membranes of rabbit skeletal muscle reconstituted in planar lipid bilayers	Single channel current fluctuations recorded by a two electrode voltage clamp	11.4 12.5	1 5	7.0	20-22	Lattore, Vergara & Hidalgo (1982)

among different preparations, may therefore be real ones. The study of channels reconstituted in artificial lipid bilayers where the composition of the lipid component is under the experimenter's control, will undoubtedly provide further insight as to the nature of these differences.

Equation (1) was derived (Ehrestein, Lecar & Nossal, 1970) by assuming that the channels exist in either an open or a closed state and that the relative numbers in each state are dictated by the Boltzman distribution law. The effect of increasing the Ca^{2+} concentration is to shift the curve, described by Equation (1), along the voltage axis towards more negative potentials without significantly affecting its shape. According to Wong, Lecar and Adler (1982) a tenfold increase in the free calcium concentration shifts the curve by 65mV in Ca^{2+}-activated K^{+}-channels of cloned anterior pituitary cells. They explain this by assuming that the calcium binding step is voltage sensitive and highly co-operative (i.e. channel opening is affected only if a number of calcium ions bind simultaneously to receptor site(s)). Following this line of analysis they suggest a model (see appendix 2 for discussion of this model) whereby channel opening requires the simultaneous binding of 3 calcium ions. The interpretation, that channel opening requires the co-operative action of three calcium ions is in agreement with the conclusion

reached by Meech and Thomas (1980) following their analysis of the relationship between the quantity of injected calcium and the amplitude of the resulting hyperpolarization in snail neurones. Recent kinetic studies of Ca^{2+}-activated K^+-channels in rat myoballs (Methfessel & Boheim, 1982) and of Ca^{2+}-activated K^+-channels extracted from rat skeletal muscle and reconstituted into planar lipid bilayers (Moczydlowski & Lattore, 1983) also suggest that at least two calcium ions are needed to open a K^+ channel.

On the other hand Gorman and Thomas (1980), based on their observations that the Ca^{2+}-activated K^+ conductance varies linearly with the intracellular free calcium ion concentration and that it increases e-fold for a 25mV change in holding potential, proposed a model which suggests that each K^+ channel is opened by the binding of a single calcium ion. Their model differs from the schemes proposed by Wong et al. (1982), Methfessel & Boheim (1982) and Moczydlowski & Latorre (1983) in that it assumes that the voltage dependence of the Ca^{2+}-activated K^+ conductance is caused by a rise in the calcium ion concentration close to the receptor site located halfway through the membrane whereas in the latter models the voltage dependence of the K^+ conductance resides in the binding reaction between calcium ions and their respective receptor sites. Gorman and Thomas (1980) have in fact pointed out that an alternative interpretation for the voltage dependence of the Ca^{2+}-activated K^+ conductance

can be achieved by attributing voltage dependence on the binding step between a calcium ion and its receptor site rather than a local increase in the calcium ion concentration near the receptor site.

Section 4 : Activation of K^+-channels by ions other than calcium

With the exception of magnesium, a number of other divalent ions can mimick the action of calcium. Orskov (1935) and later Passow (1981) demonstrated that lead ions (Pb^{2+}) could substitute for calcium ions in enhancing K^+ efflux from red blood cells. Passow showed that Pb^{2+} evoked K^+ channel activation by binding to the same receptor site(s) on the channel protein normally occupied by calcium ions.

Meech (1974α) found that whereas strontium ions (Sr^{2+})and barium ions (Ba^{2+}) could both activate K^+-channels in Aplysia neurons, magnesium ions (Mg^{2+}) were totally ineffective in opening K^+ channels. In a later study, however, Meech and Thomas (1980) re-examined the effect of barium injections and concluded that the calcium sensitive receptor of the K^+ channel is not sensitive to barium. They attributed the hyperpolarizing effect sometimes produced by barium ions to the displacement of calcium ions from intracellular sites which subsequently evokes K^+ channel opening.

Patch clamp recordings by Iwatsuki (1984) on

excised inside-out patches of pig pancreatic acinar cells show that at similar intracellular concentrations of calcium and barium (10 & 3uM respectively), barium is far less effective in opening K^+ channels than calcium, at all potentials tested.

Vergara and Lattore (1983) working on Ca^{2+}-activated K^+-channels from rabbit skeletal muscle incorporated into planar lipid bilayers reported that in the presence of 1-10uM ionized free calcium concentration on the cis (cytoplasmic side of the membrane in vivo) side of the membrane, addition of barium in the concentration range of 1-80μM did not enhance the open state probability of the channels. They therefore concluded that barium cannot by itself promote K^+ channel opening.

Section 5 : Inhibitors of Ca^{2+}-activated K^+ channels

There is as yet no single compound that can be regarded as a specific inhibitor of the Ca^{2+}-activated K^+-channel in the sense that tetrodotoxin (TTX) is for Na^+ channels. Several organic compounds and inorganic cations have shown promise but their effects vary from preparation to preparation. For example bath application of tetraethylammonium (TEA) ion blocked the Ca^{2+}-activated K^+ current in bullfrog sympathetic neurones (Adams et al., 1982) with full inhibition achieved at a concentration of 5mM. On the other hand, both the spontaneous and the electrically evoked hyperpolarizing responses observed in mouse fibroblasts and generated by the opening of Ca^{2+}-activated K^+-channels (see Table 1 in section 2) were only fully suppressed by external TEA at a concentration of 100mM (Okada, Roy, Tsuchiya, Doida & Inouye, 1977). Interestingly, in the same preparation, the nonyltriethylammonium ion a more hydrophobic analogue of TEA, blocked hyperpolarizing responses at a bath concentration of only 0.5mM(Okada,Tsuchiya & Yada,1982)

Exposure of the cytoplasmic membrane side to TEA also lead to blockade of Ca^{2+}-activated K^+-channels as reported by Wong et al. (1982) in cloned anterior pituitary cells. Again a relatively high concentration (20mM) was needed in order to achieve channel block. Oligomycin, a cationic ionophore, has been shown to promote a reduction in the affinity of the binding site

for Ca^{2+} in human red blood cells. It also brought about a decrease in the maximal rate of K+ efflux (Porzig, 1977).

The potential sensitive carbocyanine dyes used as fluorescent probes of membrane potential also seem to exert an inhibitory effect on the Ca^{2+}-activated K^{+} efflux in suspensions of resealed red cell ghosts (Hlady and Rink,1976). Simons (1976,1979) also reports a 50% inhibition by these agents of the Ca^{2+}-mediated K^{+} transport in human red cell ghosts at concentrations of 20-50uM. Their effect, however, seemed to depend on the external potassium concentration and they lost their inhibitory properties when the external potassium concentration was higher than 2mM. The mechanism of action of these dyes is at present unclear.

Another family of organic compounds, namely the quinines and quinidines, have also been found to interfere with the Ca^{2+}-stimulated K^{+} efflux in human red blood cells (Armando-Hardy , Ellory , Ferreira , Fleminger & Lew , 1975). Half maximal inhibition was observed at concentrations of 0.1-0.2mM, the effect being readily reversible. Also Puil and Krnjevic (1977;see page 261 of the review by Lew & Ferreira,1978) have reported a suppressing effect of quinine on the post-spike hyperpolarization observed in cat lumbosacral motorneurons, which these workers attributed to an activation of a Ca^{2+}-mediated K^{+} permeability. Quinine and quinidine also blocked the hyperpolarizing component of the light response of barnacle photoreceptors (Hanani & Shaw, 1977).

In pancreatic B-cells a characteristic pattern of spike activity was obtained upon glucose stimulation (Atwater, Dawson, Ribalet & Rojas, 1979). The pattern consisted of an initial depolarization that initiated a burst of action potentials and this was followed by a silent period during which the membrane potential was restored to its original level. External application of quinine (100uM) in the absence of glucose caused membrane depolarization (10-30mV) and within minutes an irregular pattern of spike discharge ensued. To account for the action of quinine Atwater et al. (1979) suggested that in the absence of glucose the level of the resting potential was set predominantly by a Ca^{2+}-activated K^+ conductance. They further suggested that quinine by blocking this conductance depolarized the membrane, thus initiating spike activity. In the presence of glucose, on the other hand, bath application of quinine abolished the silent phases which Atwater et al. (1979) attributed to inhibition by quinine of the Ca^{2+}-activated K^+ conductance. These compounds are known to inhibit the action of calmodulin (Lackington & Orega, 1981) which might suggest a role of this powerful regulatory protein in modulating the Ca^{2+}-activated K^+ conductance.

Of the inorganic agents, barium blocks a variety of K^+ channels (Standen & Stanfield, 1978; Armstrong & Taylor, 1980; Eaton & Brodwick, 1980). Recently Iwatsuki (1984) has shown, using the patch clamp

technique in the whole cell recording mode, that external barium at a concentration of 5mM reduced the Ca^{2+}-activated K^+ current found in pig pancreatic acinar cells. His observations are in agreement with earlier results by Eckert and Lux (1977) and by Connor (1979) which demonstrated a great suppression by external barium of the Ca^{2+}-activated K^+ current found in molluscan neurons. Strontium was also reported by these workers to exert a similar suppression but its effect relative to barium was a lot less .

Ca^{2+}-activated K^+-channels isolated from rabbit skeletal muscle and reconstituted into planar lipid bilayers have also been reported to undergo blockade by barium (Vergara & Lattore ,1983). The efficacy of the block was greater when barium was added on the cis (intracellular) side. Only micromolar amounts were needed to induce blockade from the intracellular side whereas millimolar amounts were required for the block to occur from the trans (extracellular) side . A similar internal block of a Ca^{2+}-activated K^+ conductance by barium ions has also been reported in molluscan neurones (Gorman & Hermann, 1979).

Section 6 :Possible modulators of Ca^{2+}-activated K^+ - channels.

De Peyer, Cachelin,Levitan & Reuter (1982) recently reported that addition of the catalytic (C-sub unit) subunit of cAMP-dependent protein kinase to the cytoplasmic side of snail neurones increased Ca^{2+}-

activated K^+-currents. Recently Ewald, Williams and Levitan (1985) investigated at the single channel level, the effects of the C-subunit on Ca^{2+}-activated-K^+ channels found in the F-cluster neurones of the land snail, Helix aspera. Their results show that intracellular application of the C-subunit at concentrations of 0.4μM or less enhanced channel activity by increasing the product Np, where N is the number of active channels in the patch and p is the open state probability of an individual channel. These workers have therefore proposed that the activity of individual Ca^{2+}-activated K^+-channels is modulated by cAMP-dependent protein phosphorylation. This is reminiscent of the action of cAMP on voltage sensitive calcium channels where again phosphorylation of the channel is thought to govern some aspects of their activation (see review by Reuter,1983).

Another possible modulator is intracellular pH or rather changes in intracellular pH. Cook, Ikeuchi and Fujimoto (1984), working on identified rat pancreatic B-cells at the single channel level, have demonstrated the existence of channels with a single channel conductance of about 240pS (see Table 1) that are activated by intracellular concentrations of free calcium greater than 1uM. Although they have not carried out ionic substitution experiments in order to determine the selectivity of the channel towards different cations, they have tentatively identified it as a Ca^{2+}-activated K^+-channel, presumably from its

conductance characteristics. These workers further reported that at any given voltage, and at a fixed intracellular ionized free calcium concentration, the open state probability of the channel shifts towards more positive potentials along the voltage axis by acidification of the cytoplasmic membrane surface.

In pancreatic B-cells, Ca^{2+}-activated K^{+}-channels are thought to conduct the dynamic pacemaker current (Atwater et al., 1979) which helps to terminate periods of spike activity (induced by exposure of cells to glucose) thus repolarizing the membrane and producing the quiescent periods observed in between bursts of action potentials. It is also known from the work of Malaisse et al. (1979) that during glucose stimulation the intracellular concentration of H^{+} rises. By combining these observations with their own Cook et al. (1984) have proposed that a fall in intracellular pH during glucose stimulation probably causes inhibition of the pacemaker current which in turn leads to a prolongation of the spiking plateau phase and a shortening of the silent phase.

Ginsborg, House & Silinsky (1974) have shown in cockroach salivary glands that bath application of the neurotransmitter dopamine elicited a marked hyperpolarization, caused by a selective rise in the membrane conductance to K^{+} ions. Later on Ginsborg,House and Mitchell(1980) provided indirect evidence (calcium readmission response) which suggested

that a calcium influx caused acinar cells to hyperpolarize. Exposure of cockroach salivary glands to the ionophore A23187 was found also to be effective in evoking acinar cell hyperpolarization (Mitchell & Martin 1980). Both of the above observations lend support to the hypothesis that the hyperpolarizing action of dopamine is mediated through a rise in the intracellular concentration of free calcium. Ca^{2+}-activated K^{+}-channels have been identified through the use of the patch clamp recording method in mammalian salivary glands (Maruyama,Gallacher & Petersen, 1983) . It is very likely that this is the case in cockroach salivary glands. If this is so then interesting questions arise regarding the mechanism of dopamine action which leads to an enhancement in the membrane conductance to K^{+}. A similar effect of dopamine has also been demonstrated in mammalian hippocampal pyramidal neurones (Benardo & Prince, 1982). Again external application of the transmitter was found to evoke a long lasting hyperpolarization (of the order of 10sec) which these workers attributed to a Ca^{2+}-induced K^{+} permeability change. This is supported by their additional observation that intracellular injection of EGTA into these neurones blocked the dopamine induced hyperpolarization.

The polypeptide hormone cholecystokinin (CCK) has recently been shown (Maruyama & Petersen ,1984) to markedly enhance the Ca^{2+}-activated K^{+} conductance of pancreatic acinar cells. Furthermore a sustained CCK

evoked increase of outward K^+ current required the presence of external calcium. In order to explain this, Maruyama and Petersen suggested that a sustained elevation of intracellular free calcium (which would in turn lead to a sustained outward K^+ current) could also be achieved through continuous calcium influx across the cell membrane, facilitated by CCK. Laugier and Petersen (1980) have already demonstrated electrophysiologically an apparent enhancement of the acinar cell membrane permeability to calcium ions upon sustained exposure of the tissue to acetylcholine (Ach). The effects caused by application of acetylcholine to pancreatic acinar cells are indistinguishable from those elicited by CCK. However, no explanation was given for the mechanism of action on the apparent calcium permeability.

The action of substances that are not normally found under physiological conditions but are nevertheless useful experimental tools in manipulating intracellular calcium buffering, must also be briefly considered. It has already been mentioned that general metabolic inhibitors, such as dinitrophenol and iodoacetate, both potentiate the Ca^{2+}-activated K^+ conductance. Evidence exists to suggest that they achieve this by interfering with the processes of calcium uptake by mitochondria (Vasington & Murphy, 1962) and presumably by other organelles that could function as calcium reservoirs. The family of

methylxanthines, and in particular caffeine, have also been shown by Kuba et al. (1972), as mentioned in Table 1, to cause rhythmic hyperpolarizations in sympathetic ganglion cells. Kuba (1980) later provided more evidence to support the hypothesis that these hyperpolarizations resulted from a selective increase in the K^+ conductance brought about by an intracellular calcium rise.

Section 7 : The functional role of Ca^{2+}-activated K^+ - channels.

The role of Ca^{2+}-activated K^+-channels is probably best understood in excitable cells that exhibit bursting behaviour associated with pacemaking. Perhaps activation of Ca^{2+}-activated K^+-channels is more important in such cells, particularly in those cells where voltage dependent inactivation of calcium channels is absent. For example voltage clamp studies of Helix pomatia neurones (Eckert & Lux, 1976) have revealed the existence of a slowly activating inward calcium current that showed almost no inactivation even after depolarizations lasting 0.5sec. Thus during a train of action potentials the opening of Ca^{2+}-activated K^+-channels could help repolarize the membrane, hence preventing excessive calcium accumulation in the cell.

In Aplysia neurones calcium influx during a train of action potentials (Meech, 1974b) caused the development of a prolonged outward potassium current which shut off activity by hyperpolarizing the membrane. Meech (1978) suggested that this modulation of electrical activity constitutes a form of adaptation. Indirect evidence also exists (see section 6 headed Modulation of Ca^{2+}-activated K^+-channels) that Ca^{2+}-activated K^+-channels perform a similar function in pancreatic B-cells.

Barret and Barret(1976) have shown that opening of Ca^{2+}-activated K^+-channels might also exert a

modulatory role in the discharge pattern of frog motoneurones. In these cells, the depolarizing phase of a single action potential was partly generated by a sodium influx and partly by a calcium influx. Cessation of spike activity in these motoneurones was followed by a prominent after-hyperpolarization, a major component of which depended on the presence of external calcium. Barret & Barret assigned this component to the activation of a calcium mediated potassium efflux and Meech (1978) suggested that the observed differences in the discharge pattern of different motoneurone groups could be accounted for by corresponding differences in the Ca^{2+}-activated K^{+}-channels present in each group. Since the conductance of Ca^{2+}-activated K^{+}-channels seems to be fairly constant in different preparations, the parameters that are likely to differ in different motoneurone groups are the sensitivity of the channels to changes in intracellular calcium and the relative number of channels present in each group.

Ca^{2+}-activated K^{+}-channels might also help regulate secretion of catecholamine by adrenal chromaffin cells. Douglas and Rubin (1961) have shown that the acetylcholine evoked secretory response of the adrenal medulla was abolished in the absence of extracellular calcium. Moreover a quantitative relation was found between the amount of catecholamine released by acetylcholine and the extracellular calcium concentration . Recently Fenwick, Marty and Neher

(1982) have identified using the patch clamp recording technique the presence of voltage-gated Ca^{2+} channels in adrenal chromaffin cells. The existence of Ca^{2+}-activated K^{+}-channels in chromaffin cells had previously been demonstrated by Marty (1981). Their function in these cells might be to stop calcium influx through voltage-sensitive Ca^{2+} channels during secretagogue stimulation by shifting the membrane potential towards E_K.

In many fluid secreting epithelia the action of secretagogues is to promote loss of cellular potassium (Burgen, 1956; see also Petersen, 1980). The most probable pathway for this efflux is through Ca^{2+}-activated K^{+}-channels whose presence in these tissues has been directly demonstrated through the use of the patch clamp recording technique (see Table 1). Recently Maruyama and Petersen (1984) have proposed that potassium released from the basolateral membrane surface re-enters the cell via a K^{+}-Na^{+}-Cl^{-} co-transport system(situated in the basolateral membrane) thus enabling the simultaneous transport of NaCl into the cell. However, they made no attempt to link the events happening at the basolateral membrane to events occurring at the luminal membrane, where fluid secretion takes place.

Marty, Tau and Trautmann (1984) working with rat lacrimal glands provided evidence for the existence of three different kinds of channels activated by a rise

in intracellular free calcium. Big unitary conductance potassium channels (BK), with a single channel conductance of 250pS in symmetrical potassium solutions (140mM on either side of the patch) were found to be activated by a rise in the concentration of free calcium lying in the range 0.01 to 0.1μM. Elevation of the intracellular calcium concentration in the range 0.1 to 1uM effected the opening of a second class of channels preferentially selective to chloride ions. An even further increase in free calcium to values >1μM caused activation of a third class of channels identified as non-selective cation channels (25pS). Moreover a cytosolic calcium concentation of 100uM was reported by these workers to bring about blockade of BK channels at positive potentials. According to these workers both the initial and sustained KCl or NaCl secretion observed in a number of secretory tissues (Burgen, 1956; Alexander,Van Lenner & Young, 1972), can be solely accounted for by activation of these channels in conjunction with the operation of a Na^+-K^+ pump known to be present in exocrine glands (Petersen, 1973).

In summary, therefore, Ca^{2+}-activated K^+-channels probably play a vital role during the secretory process in exocrine cells, but the details of how their functioning is linked to fluid secretion remains to be elucidated.

The process of phagocytosis has also been related to a slow membrane hyperpolarization (of the order of

seconds) produced by opening of Ca^{2+}-activated K^{+}-channels. (see review by Oliveira-Castro, 1983). Kouri, Noa, Diaz and Niubo (1980) have shown that phagocytosis of latex particles by rat peritoneal macrophages is assocated with a slow membrane hyperpolarization.

Yet another proposed function of such hyperpolarizing responses is modulation of cell motility. Experiments by Gallin and Gallin (1977) have shown that macrophages responded to the application of certain chemotactic factors by undergoing transient hyperpolarizations. Okada, Tsuchiya and Inouye (1979) have suggested that the spontaneous hyperpolarizing responses observed in fibroblasts are a form of a "receptor potential" associated with cell motility. It is interesting to note at this point that a similar hyperpolarizing response has been observed in Paramecium (posterior mechanoreceptor current; see review by Eckert and Brehm, 1979) where it is thought to mediate the escape response of the cell. During the period of hyperpolarization, ciliary beat frequency was augmented thus enabling the organism to move away from the stimulus source. Since evidence exists (Satow,1977;see Table 1)for the presence of Ca^{2+}-activated K^{+} -channels in Paramecium, it might be reasonable to attribute enhanced ciliary activity during hyperpolarization to an elevation of cytosolic free calcium rather than to the potential change per

se. However, experiments on mutant strains of Paramecium, that lack calcium channels have shown that artificial hyperpolarization (current clamp) is sufficient to induce an increase in ciliary beat frequency. The possible involment of Ca^{2+}-activated K^{+}-channels in the requlation of beat frequency in Paramecium requires further investigation.

Disussion of the possible role of Ca^{2+}-activated K^{+}-channels in mammalian eggs has been reserved for the General Discussion of the thesis.

CHAPTER I : METHODS

Section 1 : Egg donors and method of collecting eggs

The egg donors were mature (6-10 weeks old) virgin female golden hamsters and mature (6-8 weeks old) virgin female mice (Balb/c strain).

Hamsters, maintained under controlled lighting (8h dark/16h light) were induced to superovulate as follows. They were first given an intra-peritoneal (IP) injection of 30 i.u. of pregnant mare's serum gonadotrophin (PMSG) (Folligon: Intervet Labs Ltd., Cambridge) in the early evening. Forty eight hours later they were injected I.P. with 45 i.u. human chorionic gonadotrophin (HCG; G-2 Sigma Chemical Co., St. Louis, USA).

The procedure for inducing superovulation in mice was slightly different. The animals were kept under a fixed light/dark cycle (8h dark/16h light). They were first injected (I.P.) with 10 i.u. PMSG in the early evening and with 10 i.u. HCG forty-eight hours later.

Hamsters or mice were killed 15-18h after the HCG injection and their oviducts were dissected out and placed in a dish containing a physiological solution (referred to as normal) at room temperature (20-22^{o}C). The composition of the normal solution is given in Table 3.

Both hamster and mouse eggs, are shed into the oviducts surrounded by a mass of follicular cells which are collectively known as the cumulus oophorus. Oviducts viewed under a bench microscope (x16), using

fairly strong illumination, become translucent thus allowing clear visualization of ovulated eggs. In order to retrieve ovulated eggs from the oviducts a small cut was made with a pair of fine scissors, close to the site where the eggs were situated. Eggs along with their cumulus oophorus were thus released into the surrounding medium containing normal solution.

The cumulus mass, including eggs, was subsequently sucked into a microcapillary pipette and transferred to a solution containing hyaluronidase (1mg ml^{-1}, Type 1-S, Sigma) for about 5-10 min. to free the eggs from their surrounding follicular cells. After enzymatic dissection of the egg mass, each egg was now left surrounded by its zona pellucida. In hamster eggs the zona pellucida was removed, by transferring eggs freed from cumulus cells into a normal Ringer solution containing trypsin (1mg ml^{-1}, Type III, Sigma) for 1-3 min. Removal of the zona pellucida of mouse eggs was achieved by transferring eggs (freed from cumulus) into a normal solution containing protease (1mg ml^{-1}, Type XIV, Sigma) for 10-15min.

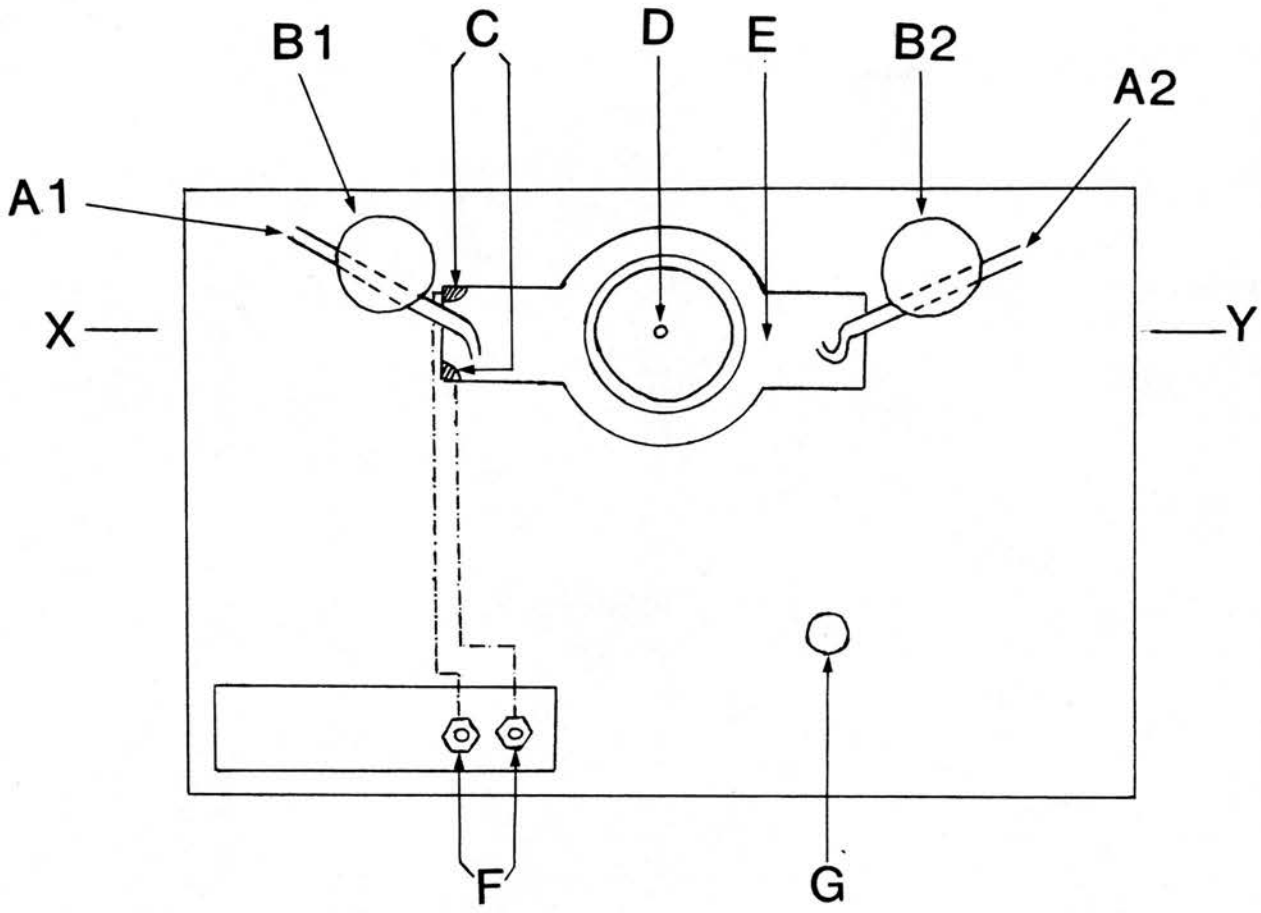
Enzyme treatments were carried out at room temperature (22-24^{o}).

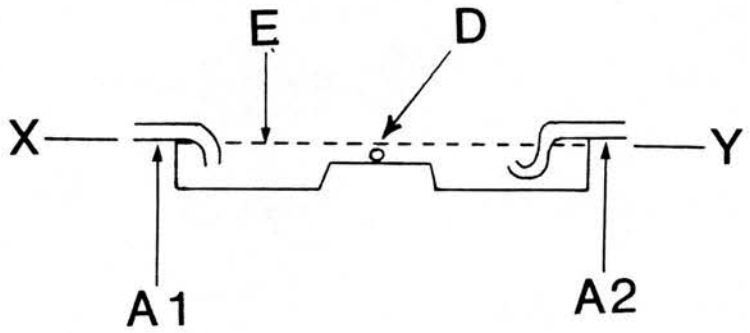
Section 2 : Intracellular recordings

An egg was transferred to a chamber (volume 5ml) mounted on an inverted microscope (x400 ; Biovert, Reichert, Austria). A schematic diagram of the experimental chamber is shown in Fig.1a .The chamber contained a solid silver/silver chloride electrode in

Figure 1a

Schematic diagram of the experimental chamber. Upper diagram shows a top view of the experimental chamber which has been drawn to scale. The arrows A1 and A2 indicate the glass capillaries used for exchanging solutions in the experimental chamber E. Capillaries A1 and A2 were connected via plastic tubing to a Watson-Marlow H.R Flow inducer (MRHE 200). The desired solution was delivered into the chamber through A1 and simultaneously an equal amount of fluid was removed through A2. The capillaries were held in place by the studs labelled B1 and B2. A pair of reference electrodes C, which consisted of solid silver chloride pellets were situated at one end of the chamber. They were connected to the terminals F. The egg, D, was placed in the middle of a transparent elevated platform which allowed illumination from below. The whole chamber was anchored to the microscope stage by screw G. The lower diagram shows a section through X-Y.


B1
C
D
E
B2
A2
A1
X
Y
F
G


E
D
X
Y
A1
A2

contact with the bathing solution. This served as the reference electrode. In some experiments described in Chapter II, where the chloride concentration in the bathing solution was changed, the bath was earthed via a 3M-KCl saline-agar bridge. Exchange of different bathing solutions was achieved by a Watson-Marlow H.R. Flow Inducer (MRHE200) which pumped solutions through the chamber at a rate of about 5ml/min.

A single microelectrode (30-70MΩ) filled with 2M K-acetate was used for simultaneous monitoring of membrane potential and input resistance. Despite the fact that the use of a single microelectrode suffers from unpredictable changes in electrode resistance upon cell impalement, it was preferred over the use of independent microelectrodes for current passing and voltage recording in order to minimize the possibility of microelectrode induced damage upon cell penetration. In some experiments, however, two separate microelectrodes were employed in order to check the reliability of the resistance measurements obtained by recording through the single microelectrode technique.

The recording microelectrode(s) was connected via a bridge circuit to the input of a high impedance pre-amplifiers. The type of pre-amplifiers used were models KS700 or M707 (input impedances of $10^{11}\Omega$ and $10^{11}\Omega$ respectively) made by W.P. Instruments, U.S.A. In a number of experiments a DAGAN (8100) single microelectrode recording system (input impedence $5x10^{11}\Omega$) was employed instead.

Cell impalement was achieved by resting the microelectrode on the surface of the egg until a slight swelling of the egg was observed. Contact with the egg was also indicated by a slight deflection of the zero voltage baseline. It was not necessary to immobilize the cell because it gently adhered to the glass base of the chamber. Penetration was effected by either overcompensation of negative capacity or by passing large hyperpolarizing current pulses (>3nA, 1sec duration, frequency of 0.3Hz) between the barrel of the microelectrode and the bath electrode by means of the bridge circuit.

Current pulses were generated by a Devices stimulator, triggered for delivery via the bridge circuit to the microelectrode by a Digitimer (D4030; Devices Ltd.).

Once inside the cell, application of hyperpolarizing or depolarizing current pulses produced passive electrical responses (electrotonic potential), which were monitored for the determination of the cell's input resistance and time constant. The recording microelectrode was always balanced prior to cell impalement. In general either a slight (1-3mV)or no imbalance in the bridge circuit was detected following cell impalement, indicating only a small or no increase in electrode resistance. Recordings where a large bridge imbalance (>3mV) was detected were abandoned.

Attempts to measure the absolute value of the

membrane potential of cells which have high input resistance, requires that the input bias current (leakage current) of the recording pre-amplifier is either minimized (preferably to values below 5pA) or measured and accounted for by substracting its contribution to the observed membrane voltage.

The leakage currents of the WPI preamplifiers were measured with the aid of a 20MΩ in-built resistor and adjusted to less than 5pA. Sometimes an external resistor of 100MΩ was used to monitor and adjust the leakage current. Using again an external 100MΩ resistor the leakage current drawn by the DAGAN (8100) pre-amplifier was found to be 15pA. No adjustment of leakage current was carried out and the membrane potentials were corrected as indicated above. In experiments (marked by an asterisk in Table 7 in Part II of Chapter II) where the DAGAN preamplfier was used to record changes in membrane potential induced by injections of Ca^{2+}, the effect of the 15pA leakage current on the recorded membrane potential was ignored. This is because after the insertion of the coarse Ca^{2+} microelectrode the input resistance only occasionally exceeded 200MΩ with the result that any error in the measurement of the membrane potential introduced by the 15pA amplifier leakage current would amount only to a few millivolts (the IR drop produced by 15pA flowing through 200MΩ is 3mV).

During the course of the project one of the WPI

amplifiers (model KS700)) developed a fault, namely the development of a large (20-100pA) leakage current. This current flowed in the direction to hyperpolarize cells and its amplitude was proportional to the input resistance of the source, becoming prominent at input resistances exceeding 200MΩ. Results obtained with the faulty amplifier are not presented in this thesis although some were unfortunately published in two papers (Georgiou, et al., 1983 and Georgiou et al., 1984) before the fault was detected. These papers are appended to the thesis.

Permanent experimental records were obtained as pen recorder traces on a Devices M2 Recorder or as photographs of the screen of a storage oscilloscope (RM5113, Tektronix Ltd.).

Section 3 : Calcium Injections

After a K-acetate microelectrode had been inserted into the eggs and stable recordings of membrane potential and input resistance were obtained, a second microelectrode filled with 1M $CaCl_2$ (10-15MΩ when filled with KCl) was subsequently lowered on the egg surface until sufficient pressure was exerted to cause a slight but detectable increase in the diameter of the egg. Hyperpolarizing current pulse (>10nA) from a Devices stimulator were delivered via a 1GΩ resistor, in series with the barrel of the calcium microelectrode and a second bath electrode until penetration was achieved.

Calcium was injected ionophoretically into hamster

or mouse eggs by passing depolarizing current pulses across the cell membrane. The membrane response to a calcium injection was monitored by the K-acetate microelectrode. The charge-transfer through the calcium-injecting microelectrode lay in the range of 5 to 100nC. The amount of calcium (Ca^{2+}) injected by an ionophoretic injection is given by the following equation:

$$\triangle [Ca2+] = \frac{itn_{Ca}2+}{zF} \quad \text{..... equation 1}$$

where i is the current, t is the time (sec), z is the algebraic valence of the calcium ion, and F is the Faraday constant and $n_{Ca}2+$ is the transport number of Ca^{2+} (i.e. the fraction of the current carried by Ca^{2+}). The transport number reported by different workers varies from 0.11 (Kusano, Miledi & Stinnakre, 1975) to 0.3 (Gorman & Thomas, 1980).

For i = 5nA, t = 1sec and $n_{Ca}2+ = 0.3$

$$\triangle [Ca^{2+}] = \frac{5 \times 10^{-9} \times 0.3}{2 \times 96,500} = 7.77 \times 10^{-15} \text{mol}$$

$$= 7.8 \text{ fmol}$$

The expected rise in the average intracellular $[Ca^{2+}]$ in a spherical cell of 40um in radius (excluding the unknown value contributed by the numerous microvilli protruding from the egg surface) that would be achieved by the transfer of 7.8 fmol of Ca^{2+} is about 2.9μM. For i = 100nA, t = 1 sec and $n_{Ca}2+ = 0.3$ $[Ca^{2+}]_i$ would rise by 580μM above its resting level. These theoretical estimates of $[Ca^{2+}]_i$ ignore the effect of

Ca^{2+}-buffering and extrusion mechanisms in counteracting the increase in free $[Ca^{2+}]_i$ produced by ionophoretic Ca^{2+}-injections. The considerable involvement of the various Ca^{2+}-regulating systems in limiting intracellular rises in the free $[Ca^{2+}]$ following ionophoretic injections of Ca^{2+} has recently been investigated by Hunter (1984) in mouse oocytes. After ionophoretically injecting eggs with the metallochromic calcium-sensitive dye Arsenazo III (whose absorbance spectrum changes in response to changes in free $[Ca^{2+}]$, he found that an intracellular Ca^{2+} current pulse of 100nA produced a change in the dye's absorbance spectrum equivalent to a rise in the free cytosolic $[Ca^{2+}]$ of 0.6 to 3.2μM. In theory, according to Hunter's calculations (who used a value of 0.11 for $n_{Ca}2+$) a rise of 250μM in the cytosolic free $[Ca^{2+}]$ would be anticipated by a 100nC Ca^{2+} injection. He concluded that 98.7-99.8% of the injected Ca^{2+} is rapidly bound. Hunter's results demonstrate that theoretical estimates of rises in intracellular free $[Ca^{2+}]$ achieved by ionophoretic Ca^{2+}-pulses can be misleading since they do not take into account the contribution made by intracellular sequestering organelles and by membrane pumps in removing much of the injected Ca^{2+}.

Section 4 : Solutions

The composition of solutions used for the experiments described in Chapter II is given in Table 3 The composition of solutions used for Chapters III and

Table 3 Composition of bathing solutions used in Chapter II

Solution	Composition of Solutions								
	K^+	Na^+	Cl^-	Ca^{2+}	Mg^{2+}	Lactate	Pyruvate	Proprionate	Glucose
Normal	5.0	143.5	135.4	4.0	1.2	20.0	1.0	-	5.6
1mM K^+	1.0	143.5	131.4	4.0	1.2	20.0	1.0	-	5.6
15mM K^+	15	133.0	138.4	4.0	1.2	20.0	1.0	-	5.6
25mM K^+	25	123.5	135.4	4.0	1.2	20.0	1.0	-	5.6
40mM K^+	40	108.5	135.4	4.0	1.2	20.0	1.0	-	5.6
125mM K^+	125	21	135.4	4.0	1.2	20.0	1.0	-	5.6
Cl^- -reduced	5	143.5	12.4	4.0	1.2	20.0	1.0	120	5.6

Concentrations expressed as mM. All solutions contained 5mM HEPES plus 2.5mM-NaOH to give pH7.2

When lanthanum solutions were used, the required amount of lanthanum was added to normal solution.

Table 4. Composition of bathing solutions used in Chapter III

Solution	K^+	Na^+	Ca^{2+}	Mg^{2+}	Ba^{2+}	Cl^-	Lactate	Pyruvate	Glucose
Normal	5	143.6	4	1.2		135.4	20	1.1	5
High Calcium (20mM)	5	123.6	20	1.2		147.4	20	1.1	5
High Magnesium (20mM)	5	123.6	4	20		153	20	1.1	5
High Barium (20mM)	5	123.6	4	1.2	20	155.4	20	1.1	5
High Barium (10mM)	5	123.6	4	1.2	10	135.4	20	1.1	5

Concentrations expressed as mM. All solutions contained 5mM HEPES plus 2.5mM-NaoH to give pH7.2. When lanthanum solutions were used, the required amount of lanthanum was added to normal solution.

Table 5

COMPOSITION OF SOLUTIONS USED IN CHAPTER IV

Solution	K^+	Na^+	Li^+	Choline	TEA	Ca^{2+}	Mg^{2+}	Cl^-	Lactate	Pyruvate	Glucose
Normal	5	143.6	-	-	-	4	1.2	135.4	20	1.1	5
Na-reduced (using choline)	5	3.6	-	143.6	-	4	1.2	135.4	-	1.1	5
TEA (20mM)	5	123.6	-	-	20	4	1.2	135.4	20	1.1	5
TEA (10mM)	5	123.6	-	-	10	4	1.2	125.4	20	1.1	5
Na-free (using lithium)	5	3.6	120	-	-	4	1.2	135.4	-	1.1	5
Lithium (using TEA)	5	3.6	120	-	20	4	1.2	150.4	-	1.1	5

Concentrations expressed as mM. All solutions contained 5mM HEPES plus 2.5mM-NaoH to give pH7.2.

Composition of solutions used.

IV are given in Tables 4 and 5 and respectively.

CHAPTER II-PART I

INTRACELLULAR RECORDINGS FROM HAMSTER EGGS

INTRODUCTION

Miyazaki and Igusa (1981) first reported that the mean value of the resting potential in unfertilized hamster eggs was low, averaging -29±7mV (mean ± SD, n=120 eggs). The above workers, however, also stated in the same study that the membrane potential attained relatively high values, ranging between -40 to -50mV in 7% of the total number of measurements. In addition they observed that the more negative resting potentials tended to be associated with larger input resistances (their largest value of the resting potential being -50mV with a corresponding input resistance of 400MΩ). Based on this last observation they suggested that in most of their recordings the value of the membrane potential was underestimated as a result of substantial leakage due to microelectrode impalement. However, they did not perform any experiments designed to demonstrate the presence of a microelectrode induced leak conductance pathway, nor did they provide any details as to how the egg reacts in terms of changes in potential and resistance, following impalement by a microelectrode. It was, therefore, decided to re-examine the question of microelectrode induced leak conductance in hamster eggs, in order to assess its likely impact on the recorded membrane voltage.

RESULTS

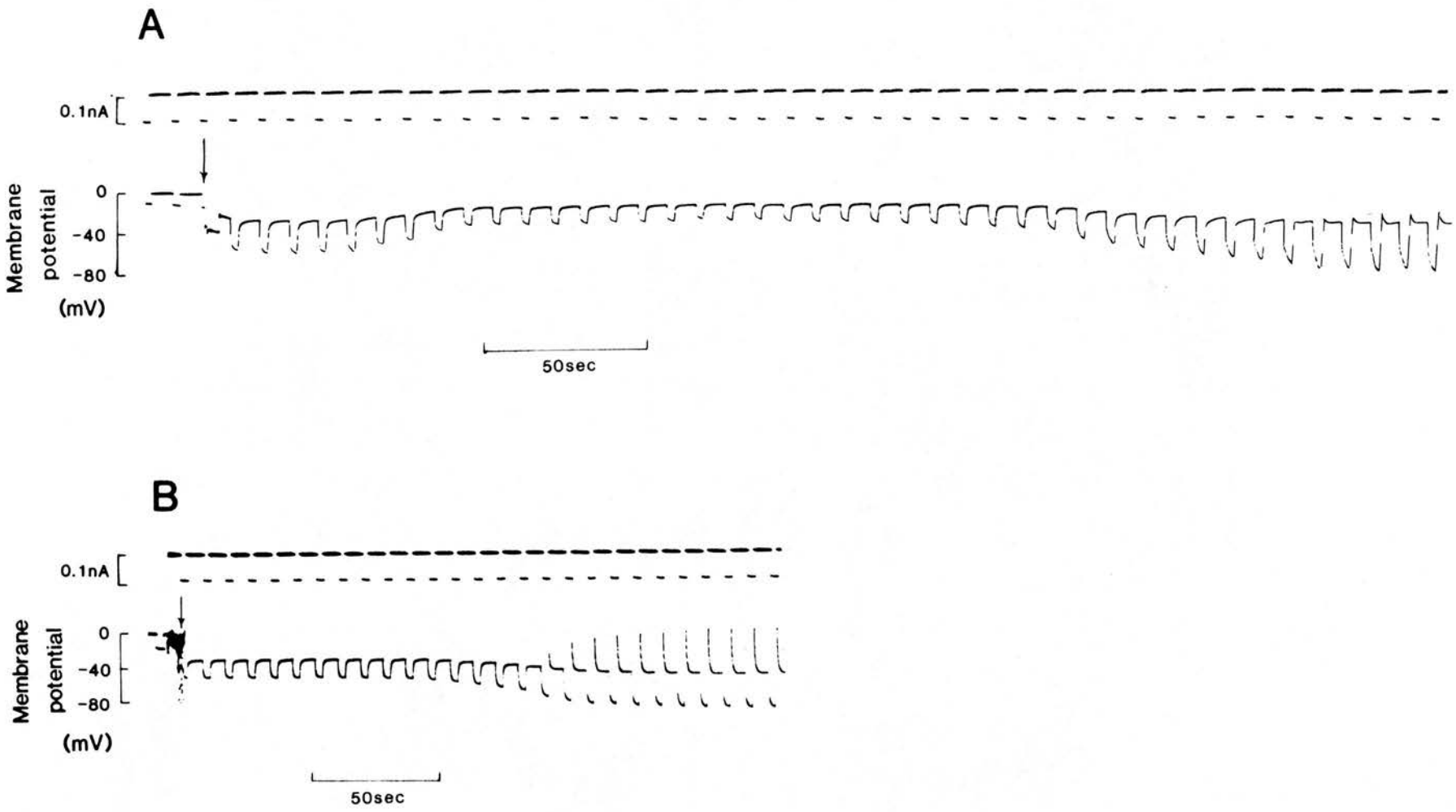
Section 1 : Intracellular recordings from hamster eggs

Figure 1b displays illustrative examples of intracellular recordings from hamster eggs using a single microelectrode. The top pen trace (labelled A), shows that just after microelectrode insertion into the egg (penetration indicated by the arrow above the voltage trace), the membrane potential declined within 50 seconds to a substantially lower and apparently steady value. This initial decline was accompanied by a corresponding large drop in input resistance, as indicated by the reduction in the amplitude of the electrotonic potentials evoked in response to the passage of a constant current pulse (shown above the voltage trace). The membrane potential and input resistance assumed low and seemingly stable values for a short period only, after which potential and resistance increased together reaching a final value of -44mV and 620MΩ respectively. The bottom pen trace (labelled B) of Figure 1b also shows a progressive rise in both potential and resistance with time but the initial decay of potential and resistance which occurs immediately after penetration of the cell by the microelectrode is not evident in this example.

The final resting potential of 36 eggs, recorded by a single microelectrode, lay in the range of -10 to -51mV, with a mean $\pm$ SD of -34 $\pm$ 9mV. (Whenever recordings were made using the DAGAN (8100)

Figure 1b

Progressive increase in the membrane potential and input resistance of two different eggs following their impalement with a microelectrode. In a typical impalement, shown in A a simultaneous decline of both resistance and potential was observed after microelectrode insertion (indicated by the arrow above the voltage trace) and then a progressive increase in potential and resistance occurred. Impalement of another egg is shown in B. The arrow above the voltage trace indicates the moment of impalement. A loss of potential upon impalement is not evident in B but the progressive rise of potential and resistance is similar to that shown in A. In both A and B the downward deflections are electrotonic potentials produced in response to constant current pulses (shown as square displacements above the voltage traces of A & B).


A
0.1nA
Membrane potential
0
-40
-80
(mV)
50sec
B
0.1nA
Membrane potential
0
-40
-80
(mV)
50sec

preamplifier the measured value of the membrane potential was corrected for amplifier leakage current as explained in the methods chapter).

The membrane resistance was determined from the linear region of the current-voltage relation. This region extended from 0 to -150mV, as observed by Miyazaki & Igusa (1982). An example of the relation between applied current and voltage response (electrotonic potential) is illustrated in Figure 2. In this particular example the final values reached by the membrane potential and the input resistance were -27mV and 170MΩ respectively.

In 36 eggs the values obtained for the input resistance range from 54 to 620MΩ with a mean $\pm$ SD of 287 $\pm$ 166MΩ. In accordance with the observations of Miyazaki and Igusa (1981) high membrane potentials were generally associated with high input resistances.

The values of the membrane potential and input resistance for each one of the 36 eggs examined are given in Table 6. Also provided in the same table are estimates of the membrane time constant and input capacitance for each individual egg. Egg diameters were measured in 17 out of the above 36 eggs, thus enabling estimation of their specific membrane resistance and capacitance. The values of these determinations are also given in Table 6.

In order to estimate the error in the measurement of the input resistance that might arise as a result of recording via a bridge circuit, six additional

Figure 2

Current-voltage relation of an egg. A shows current pulses passed through the intracellular micro-electrode and the corresponding electrotonic potentials recorded. B shows a plot of the relation between the applied current and the membrane potential of the egg.

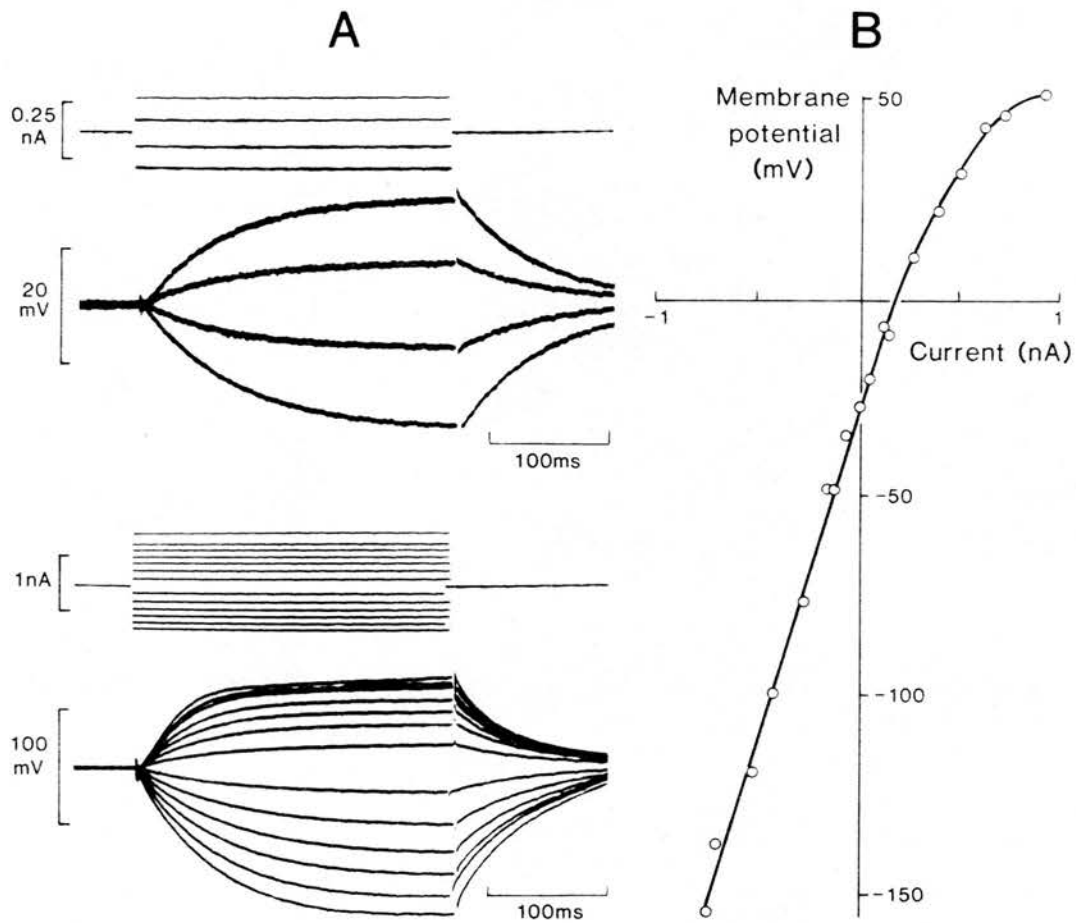

A
B
0.25
nA
20
mV
100ms
1nA
100
mV
100ms
Membrane
potential
(mV)
50
-50
-100
-150
-1
1
Current (nA)

Table 6 Passive electrical properties of unfertilized hamster eggs.

Egg No.	Resting membrane potential (mV)	Resting membrane potential corrected for amplifier leakage current (mV)	Input Resistance (MΩ)	Time Constant (ms)	Input Capacitance (pF)	Specific membrane resistance ($K\Omega cm^2$)	Specific membrane capacity ($\mu F/cm^2$)
1	-23	-	170	51	290	-	-
2	-44	-	360	240	660	-	-
3	-46	-	200	130	650	-	
4	-48	-	180	130	720	-	
5	-27	-	170	52	310	31	1.7
6	-31	-	250	63	250	46	1.4
7	-29	-	54	28	520	10	2.8
8	-28	-	100	48	460	-	-
9	-32	-	280	170	610	59	2.8
10	-29	-	110	76	690	24	3.2
11	-35	-	170	98	590	-	-
12	-43	-	130	93	700	-	-
13	-40	-	130	120	920	29	4.2
14	-28	-	96	88	920	18	4.9
15	-36	-	260	160	620	56	2.8
16	-29		100	70	690	-	
17	-17	-	78	53	680	-	-
18	-36	-	180	100	570	35	2.9
19	-44	-	190	130	650	41	3.0
20	-27	-22	365	200	540	-	-
21	-60	-51	620	400	640	-	-
22	-33	-27	380	220	580	-	-
23	-30	-23	450	320	700	-	

24	-29	-23	420	300	710		-
25	-35	-32	250	150	610		
26	-46	-41	360	180	400		
27	-41	-35	430	290	680		
28	-54	-45	620	400	640		
29	-37	-	400	270	670	-	-
30	-44	-	620	320	520		-
31	-39	-	650	340	540	-	-
32	-38	-	320	200	620	-	-
33	-26	-	300	260	870	-	
34	-41	-	440	240	540		-
35	-38	-	250	200	790		
36	-28	-	260	200	780	-	-
Mean ± SD	-36±9mV	-34±9mV n=36	287±166MΩ	177±103ms	620±152pF	35±16 $K\Omega cm^2$ n=10	3.0±1.0 $\mu F/cm^2$ n=10

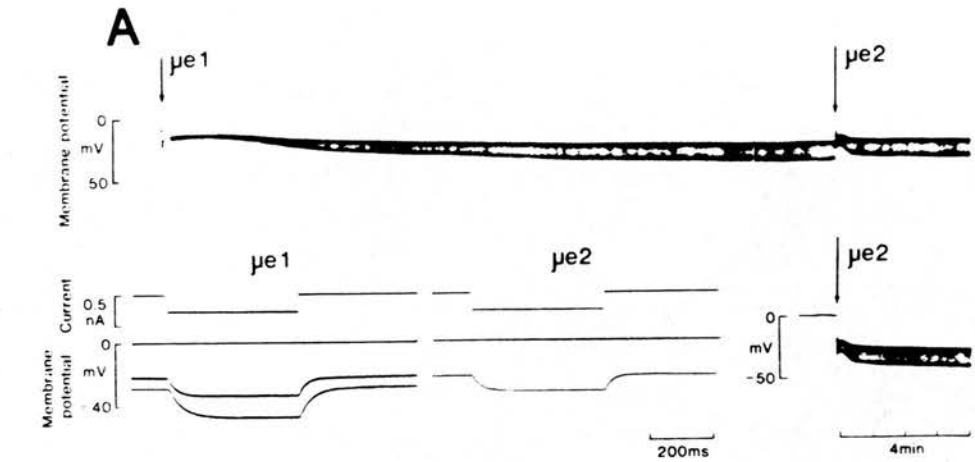
experiments were performed where two independent electrodes were used to monitor both the potential and resistance of an egg. The difference between the estimates of the cell input resistance was always found to be less than 10%. The membrane potential recorded by the two electrodes (see Fig. 3) was always within a few millivolts of each other, the small discrepancies were probably caused by differences in tip potentials of the intracellular electrodes and leakage currents of the amplifiers.

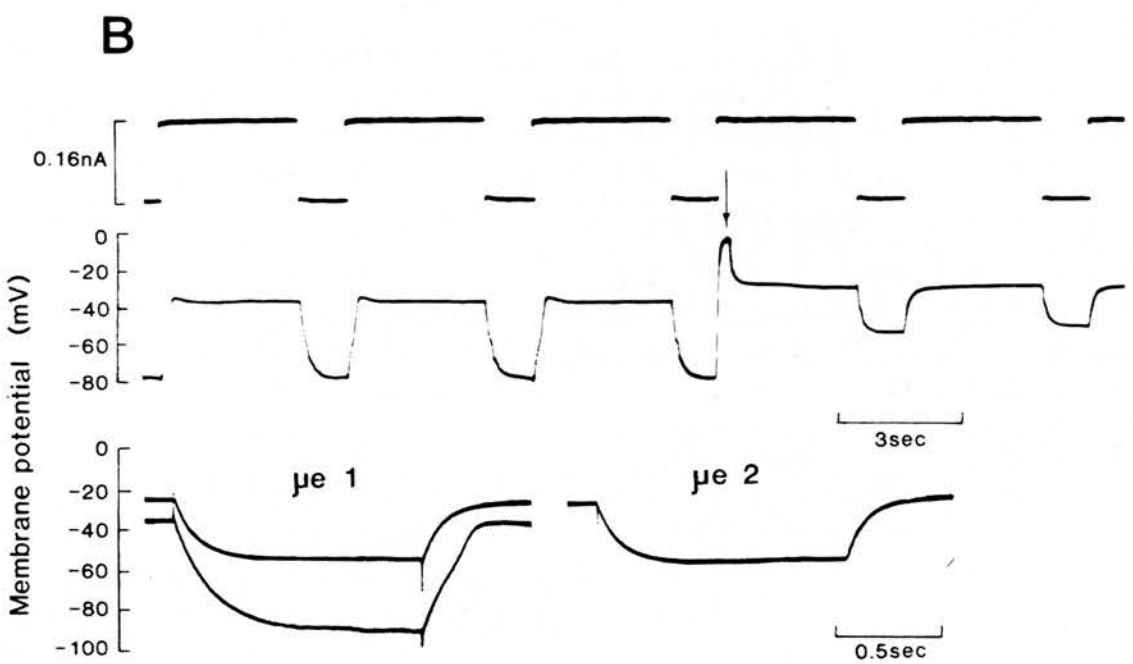
The measured mean diameter $\pm$SD of 55 eggs was found to be 79$\pm$3 μm. Given an average value of 620pF for the membrane input capacitance and an area of $2 \times 10^{-4} cm^2$ (calculated for a sphere with a radius of 40μM), the value of 3.1 $\mu F/cm^2$ is obtained for the specific membrane capacitance. This rather high value of the specific membrane capacitance is probably an overestimate since in calculating the surface area of the eggs the unknown contribution made by the numerous microvilli protruding from the egg surface could not be taken into account. If now the surface area is recalculated on the basis that the specific membrane capacity is $1 \mu F/cm^2$, typical of a lipid bilayer of 50A thick (Katz ,1966) then a value of about $6.0 \times 10^{-4} cm^2$ is obtained. This represents approximately a 3-fold increase in the surface area of an egg. By comparison, Eddy and Shapiro (1976) estimate from electron micrographs of unfertilized sea urchin eggs that the contribution made by the 2×10^5 microvilli

Figure 3

Double impalement experiments in hamster eggs. The top trace of A and the middle trace of B show the membrane potential and input resistance recorded by the first microelectrode (μe1) before and after insertion of the second microelectrode (ue2). The downward deflections (electrotonic responses) were produced by the constant hyperpolarizing current pulses passed through the first microelectrode (μe1). The lower pen trace of A shows the membrane potential and resistance recorded by the second microelectrode ; the downward deflections (electrotonic potentials) being caused by the current pulses delivered through the first microelectrode. Oscilloscope pictures of electrotonic potentials recorded by both microelecrodes are shown on the left of A. The bottom oscilloscope trace recorded by the first microelectrode (μe1) shows the electrotonic potential recorded just before the insertion of the second microelectrode (μe1) . The remaining traces were recorded seconds after impalement by the second microelectrode (μe2) . Note the large drop in input resistance recorded by ue1 at the moment μe2 was inserted in the egg (viz. 104MΩ before to 48MΩ immediatedly after impalement). A simultaneous reduction in membrane potential of about 7mV was also recorded by ue1. A second experiment of the same kind is shown in B. The middle trace of B shows the membrane potential and resistance recorded by ue1 prior to and following insertion of ue2 (indicated by the arrow).

The voltage deflections are electrotonic potentials evoked by the application, through uel, of constant current pulses (shown as square displacements in the uppermost trace). The pen trace of the voltage and the resistance recorded by μe2 is not shown. The lower trace of B shows oscilloscope pictures of electrotonic potentials recorded by both microelectrodes . The bottom oscilloscope trace recorded by μel shows an electrotonic potential prior to egg impalement by μe2. Again notice the large fall in input resistance recorded by uel immediatedly following insertion of μe2 in the egg (viz. from 250MΩ before impalement to 136MΩ just after). A concomitant reduction in membrane potential of about 10mV was also registered by uel. In both A and B the lower oscilloscope trace recorded by the first microelectrode shows the electrotonic potential recorded just before the insertion of the second microelectrode.


A
μe1
μe2
Membrane potential
0
mV
50
μe1
μe2
μe2
Current
0.5
nA
0
Membrane potential
mV
-40
0
mV
-50
200ms
4min


B
0.16nA
Membrane potential (mV)
0
-20
-40
-60
-80
3sec
0
-20
-40
-60
-80
-100
μe 1
μe 2
0.5sec

present on its surface serve to increase its surface area from $2x10^{-4}cm^2$ (calculated for a sphere of 40μm in radius) to $6x10^{-4}cm^2$.

The value obtained for the specific membrane resistance assuming an apparent surface area of $2x10^2cm^2$ and given an average input resistance of about 300MΩ(see Table 6) is approximately $66K\Omega cm^2$. This calculation again ignores the surface area contributed by the microvilli. Taking this factor into account, the specific membrane resistance becomes approximately $200K\Omega\ cm^2$. This probably represents a more realistic estimate of the specific membrane resistance but even this value might be an underestimate if the seal between microelectrode tip and cell membrane is a rather poor one. The above estimated value of $200K\Omega cm^2$ for the specific membrane resistance of hamster eggs is similar to the value of $380K\Omega cm^2$ (Jaffe & Robinson, 1976) found in sea urchin eggs. In calculating the specific membrane resistance Jaffe and Robinson have taken into consideration the increase in surface area of the egg membrane due to the presence of microvilli.

Hyperpolarizing current pulses that displaced the membrane potential beyond -80mV elicited anode-break responses as observed by Okamoto, Takahashi and Yamashita (1977) in mouse and by Miyazaki and Igusa (1982) in hamster eggs. Both of the above groups identified the anode break response as being primarily the result of a calcium influx through voltage gated calcium channels.

Section 2 : Insertion leak pathway

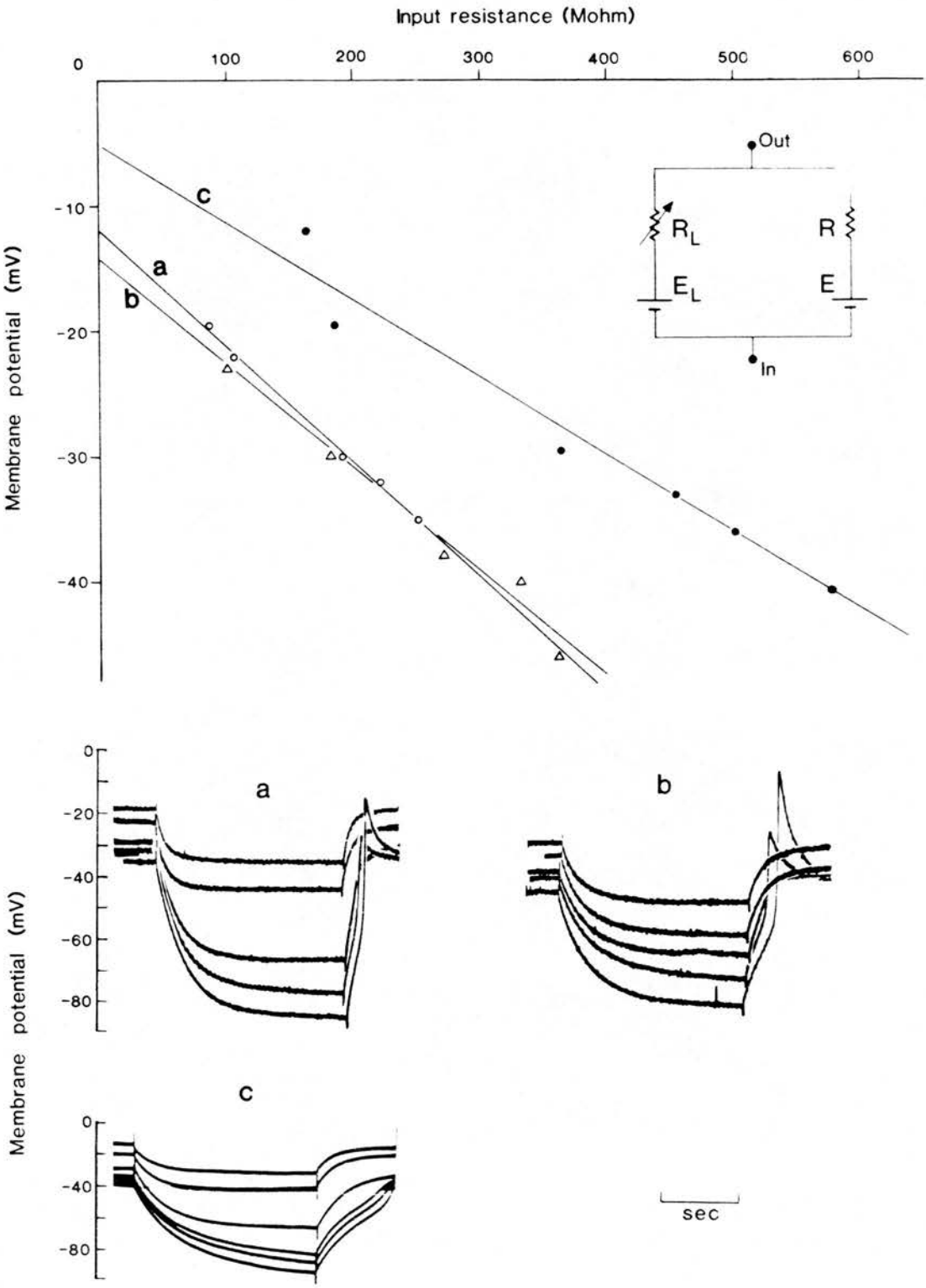
A general feature of microelectrode impalements in both hamster and mouse eggs was a parallel and progressive increase of membrane potential and membrane input resistance with time. Both membrane potential and input resistance achieved steady state values usually within 10-20 minutes. Such a temporal correspondence between potential and resistance suggests a sealing process between the membrane and the microelectrode tip which helps to reduce the conductance of the shunt pathway created during insertion. A similar gradual rise of potential with resistance has been observed in sea urchin eggs (Jaffe & Robinson ,1978 ; Chambers & de Armendi,1979) and also in Xenopus eggs (Kusano, Miledi & Stinnakre,1982 ; Dascal,Landau & Lass,1984),where it was thought to represent recovery from penetration induced damage.

Plots of apparent membrane potential against effective input resistance at various times following microelectrode impalement revealed the existence of a linear relationship between them. Examples of such plots are presented in Figure 4. The same figure also depicts an equivalent circuit model proposed to explain the observed linear relation between potential and resistance. The model assumes that the leak pathway potential, E_L , the true cell membrane potential E and the true membrane resistance R are constant, whereas the resistance of the leak pathway, R_L , is variable. According to this model the observed membrane potential

Figure 4

Progressive increase of the membrane potential and input resistance of three different eggs following their impalement with a microelectrode .The upper part shows plots of the membrane potential against the input resistance at various times during the recording. The lines were fitted by linear regression (r=0.987,0.998,0.968). The input resistance was obtained from the electrotonic responses shown in the lower part of the figure. The electrotonic potentials were elicited in response to the passage of a 0.1nA hyperpolarizing current pulse through the recording microelectrode.

The inset depicts an equivalent circuit proposed to account for the relation between resistance and potential(see text).


Input resistance (Mohm)
0
100
200
300
400
500
600
Membrane potential (mV)
-10
-20
-30
-40
c
a
b
Out
In
R_L
R
E_L
E
Membrane potential (mV)
0
-20
-40
-60
-80
a
b
c
0
-40
-80
sec

V is related to E , E_L and R' (where $R' = R_L R / R + R_L$) by the following equation.

$$V = R'(E-E_L)/R + E_L \quad \ldots\ldots\ldots(1)$$

(see derivation in Appendix 2).

Thus according to this model a plot of V against R' should be linear with a slope $(E-E_L)/R$ and an intercept of E_L.

The value of the leak battery (E_L) ranged from -2 to -14mV with a mean of -9 ± 4mV (n=9 experiments). According to the Henderson equation the expected liquid junction potential across a porous membrane separating a solution of 120mM NaCl (extracellular compartment C_1) from a solution of 120mM KCl (intracellular compartment C_2) is given by:

$$V = \frac{-RT\ [U_K - U_{Cl}]C_2 - [U_{Na} - U_{Cl}]C_1}{F\ [U_K + U_{Cl}]C_2 - [U_{Na} + U_{Cl}]C_1} \ln \frac{[U_K + U_{Cl}]C_2}{[U_{Na} + U_{Cl}]C_1} \quad \ldots.(2)$$

$$= -25 \frac{[7.6 - 7.9]140 - [5.2 - 7.9]140}{[7.6 + 7.9]140 - [5.2 + 7.9]140} \ln \frac{[7.6 + 7.9]140}{[5.2 + 7.9]120}$$

$$= -25 \frac{[-42 + 378]}{[2170 - 1834]} \ln \frac{2170}{1834}$$

$$= -3.5 \text{ mV}$$

where the ratio of the ion mobility $U_K : U_{Cl} : U_{Na}$ is 7.6:7.9:5.2 (Benedek & Villas , 1979, pp 3-17). The effective intracellular potassium concentration used was the one derived by Miyazaki and Igusa (1982) from measurements of the reversal potential of the

the calcium-evoked response in hamster eggs (see also Section 7 in Part II of this chapter). Evidently the Henderson equation fails to account for the observed magnitude of E_L . Dascal, Landau and Lass (1984) working on Xenopus eggs have calculated the expected value of the leak battery (E_L),generated at the site of microelectrode insertion,by applying the Goldman , Hodgkin and Katz (G-H-K)equation with $P_K=P_{Na}=P_{Cl}=1$. They thus obtained a value for E_L of -16mV. A value of -13mV for a microelectrode induced non-specific leak battery has been calculated by Weisenseel and Jaffe (1972) in Fucus eggs. In calculating E_L, the above workers employed a modified form of the G-H-K equation, where the ionic permeability coefficients were replaced by the relative mobilities of the major extracellular and intracellular ions, measured in sea water. A theoretical model such as the one proposed by Landau et al (1984) and by Weisenseel and Jaffe (1972), cannot at present be used to calculate the expected value of E_L in hamster eggs because little information is available regarding their intracellular ionic composition. It is likely however that once the cytosolic ionic composition of hamster eggs becomes known a model, based on a "non-specific" Goldman type equation, would be successful in producing a better fit to the experimentally determined value of E_L.

The presence of an impalement leak artifact can be identified by inserting a second microelectrode (μe 2) and observing its effect on the membrane potential and

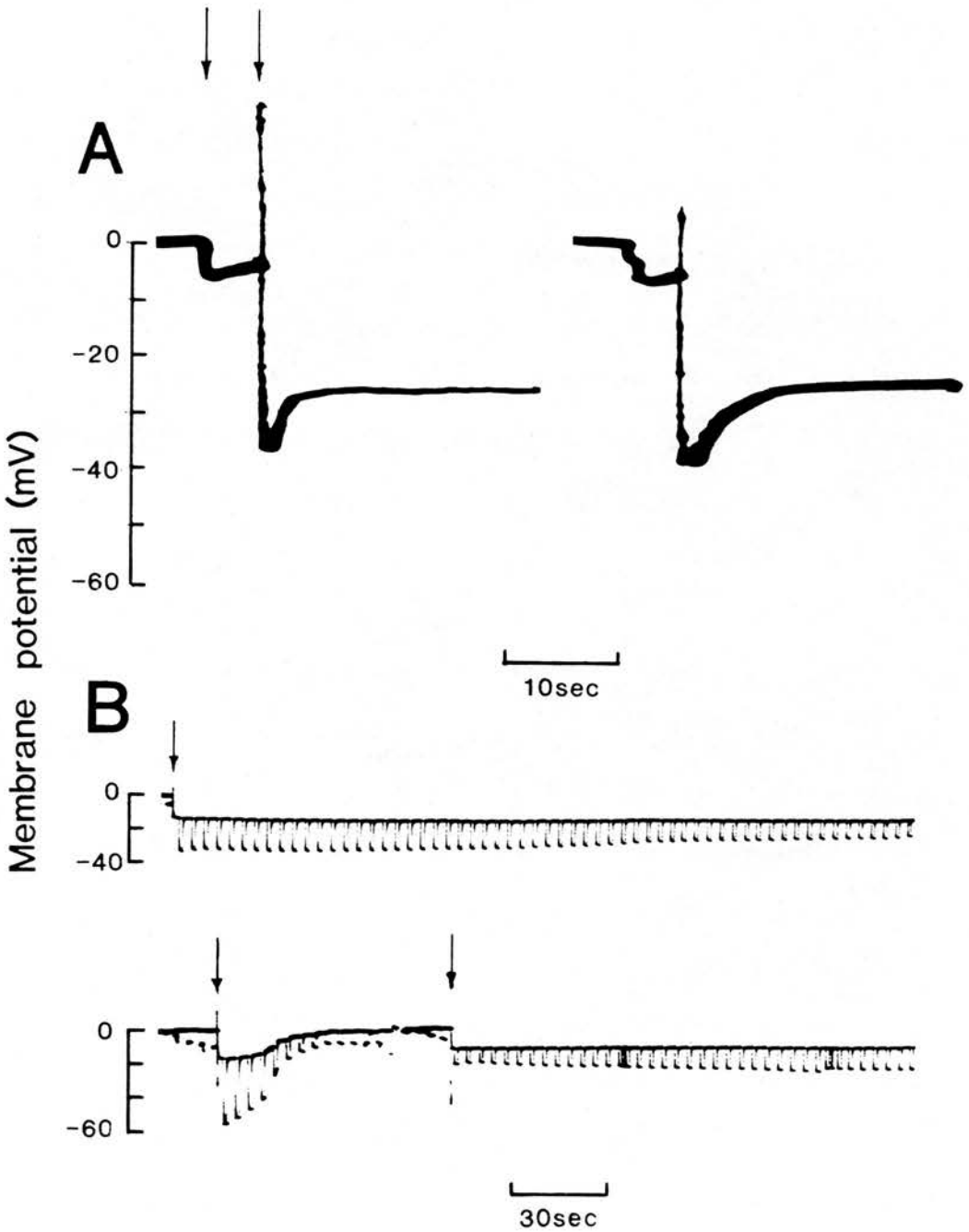
resistance recorded by a previously inserted microelectrode (μe 1). It is clear from the records of Fig. 3 that insertion of the second microelectrode caused an abrupt fall in both potential and resistance measured by the first microelectrode. The same value of the membrane potential was recorded by both microelectrodes as expected for a cell with an isopotential interior.

Another feature of the recordings already mentioned which further implies the existence of a shunt pathway is the relatively rapid decay (of the order of seconds) of the registered membrane potential just after penetration. This shunting effect of microelectrode insertion has been observed both in hamster and mouse eggs. Figure 5 shows examples of such potential decays immediately following the insertion of the microelectrode (see also Fig.1b A). Such transients have been observed in a number of other preparations (Lassen, Nielsen, Pape & Simenson, 1971; Dascal, Landau and Laos, 1984; Ince, Leigh, Meijer, van Bavel & Ypey, 1984). The general interpretation given for these events is that they represent loss of membrane potential due to an impalement leak pathway introduced at the site of the microelectrode insertion. This is further supported by the observation that the decay of the membrane voltage after microelectrode impalement was always accompanied by a reduction in the cell input resistance (also shown in Fig.5).

Figure 5

Microelectrode recordings showing decay of membrane voltage following initial egg impalement. A shows that microelectrode insertion into two different eggs is followed by a relatively rapid loss of membrane potential. Microelectrode contact with the egg caused a slight change in the baseline voltage and is indicated by the first arrow. The moment of egg penetration by the microelecrode, achieved by negative capacity overcompensation, is indicated by the second arrow. The top pen trace of B shows that loss of membrane voltage following miroelectrode insertion (arrow) is accompanied by a concomitant fall in input resistance, as judged by the monotonic reduction in the size of the electrotonic potentials evoked in response to constant current pulses (not shown). The lower pen-trace of B shows repeated impalement of the same egg. The first impalement (arrow, lower trace) was unsuccessful with the result that the microelectrode spontaneously dislodged itself from the cell. A second impalement is indicated by the arrow on the right trace.

The much lower values of the membrane potential and input resistance recorded following re-insertion probably indicates damage inflicted on the cell by the first impalement.


A
0
-20
-40
-60
Membrane potential (mV)
10sec
B
0
-40
0
-60
30sec

CHAPTER II-PART II

Ca^{2+}-ACTIVATED K^{+}-CHANNELS IN MAMMALIAN EGGS

INTRODUCTION

Microelectrode recordings during fertilization of hamster eggs *in vitro* (Miyazaki & Igusa, 1981) revealed that sperm-egg fusion was closely associated with a transient hyperpolarization of the membrane potential. The moment of sperm-egg fusion was identified as being the instant after sperm attachment at which the tail of the sperm became immotile and straight. Miyazaki and Igusa further observed that the initial sperm-induced hyperpolarization was subsequently followed by a series of similar hyperpolarizations occurring with a definite periodicity, each hyperpolarization lasting about 14 seconds. In addition the membrane input conductance rose dramatically during the peak phase of these hyperpolarizations. In a subsequent study Miyazaki and Igusa (1982) found that by injecting calcium ions into eggs they could elicit membrane hyperpolarizations resembling closely the ones produced by the action of sperm. Moreover, by carrying out ionic substitution experiments they showed that both sperm-and calcium-evoked responses were sensitive to changes in external potassium and estimated the reversal potential to shift from -85mV at $[K]_o$=5mM to -48mV at $[K]_o$=20mM. Miyazaki and Igusa therefore proposed that the sperm-induced hyperpolarizations were caused by opening of potassium selective membrane channels mediated by a rise in the cytosolic calcium activity. Although this

explanation seems to adequately fit their data, an inconsistency became apparent in their results, namely their claim that the calcium-induced hyperpolarization was associated with a decrease of the membrane input conductance rather than an increase as observed for the sperm-evoked responses. It therefore seemed advisable to reinvestigate this point since if it were found to be true it would imply that the sperm and the calcium-induced hyperpolarization were generated via different mechanisms. Igusa and Miyazaki (1983) provided evidence for an increase in membrane conductance during the Ca^{2+}-evoked hyperpolarization while this work was in progress.

RESULTS

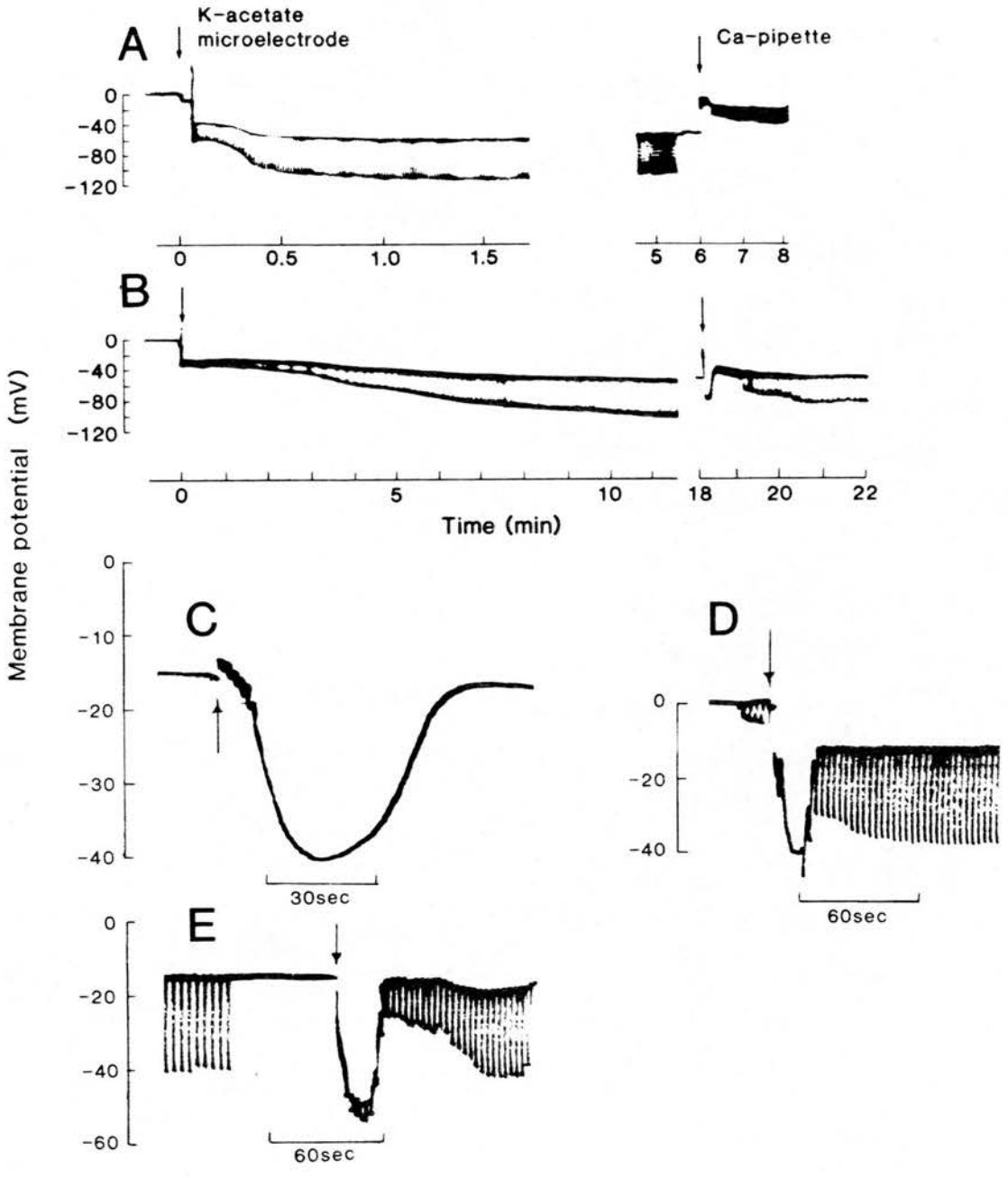
Section 3 : The effect of calcium injections in hamster eggs

The membrane response to the insertion of a calcium-containing micropipette was monitored by a K-acetate microelectrode that had already been inserted in the egg. Impalements by a calcium micropipette that caused a permanent fall in both potential and resistance (see fig.6A) were regarded as unsatisfactory and were abandoned. In general, satisfactory impalements were characterized by a transient membrane hyperpolarization, as shown in Fig.6B. This insertion-induced hyperpolarization was always associated with a prominent transient fall in input resistance. The time course of the input resistance transient always

Figure 6

Transient hyperpolarizations in hamster eggs. A shows a pen trace of the membrane potential and resistance recorded via a K-acetate microelectrode . The arrow on the left indicates the time at which the microelectrode was inserted into the egg. The downward deflections (electrotonic potentials) were produced by constant current pulses passed through the same K-acetate microelectrode. At the second arrow a calcium pipette was inserted into the egg but failed to seal as judged by the irreversible fall of both potential and resistance. B shows the results of another similar experiment , in which the insertion of the calcium pipette (arrow on the right) was judged satisfactory since it was followed by a transient membrane hyperpolarization and a full recovery of the input resistance(not shown). C is another illustration of a transient hyperpolarizing response elicited by the inserting a calcium micropipette into the egg (time of impalement indicated by the arrow). D and E are examples of hyperpolarizations produced by impalement with low resistance pipettes , containing 3M KCl and 1M $CoCl_2$ respectively. The instant of impalement is again shown by the arrows placed above the voltage trace. The brief downward deflections were obtained in response to the passage of constant current pulses through the recording microelectrode. Notice the prominent fall in input resistance as indicated by the large reduction of the electrotonic potential at the

maximum of the penetration- induced hyperpolarization. The recording electrode in D was the inserted KCl electrode whereas in E it was a previously inserted K-acetate electrode.


A
K-acetate
microelectrode
Ca-pipette
0
-40
-80
-120
0
0.5
1.0
1.5
5
6
7
8
B
0
-40
-80
-120
0
5
10
18
20
22
Time (min)
Membrane potential (mV)
C
0
-10
-20
-30
-40
30sec
D
0
-20
-40
60sec
E
0
-20
-40
-60
60sec

outlasted the timecourse of the potential change. Insertion induced membrane hyperpolarizations could also be achieved by impaling eggs with micropipettes containing other solutions (e.g. $CoCl_2$, HCl, KCl). Examples of such impalement induced responses are shown in Fig.6D and 6E. They were generated in response to the insertion of low resistance (15MΩ) micropipettes containing 3M KCl and 1M $CoCl_2$ respectively.

In some experiments the calcium pipette developed poor current passing capability after a few injections even though the initial impalement was judged satisfactory. Such experiments were abandoned.

In 63 eggs a pulse (0.5 or 1.0 sec) of calcium ions caused a transient increase in membrane potential of 27±9mV (mean ± SD), from a resting value of -26±12 mV (mean ± SD) to a value of -53±11mV (mean ± SD), measured at the peak of the response (see Table 7). The duration of the hyperpolarizing response (i.e. the time taken for the membrane potential to return to its pre-response value) averaged 35±27 seconds (n=61 responses). The respective mean values of the input resistance prior to and at the peak of the Ca^{2+}-evoked hyperpolarization were 141±94 and 40±24 MΩ.

Injections of several ions other than calcium (e.g K^+, Mg^{2+}, Ba^{2+}, Co^{2+}, La^{3+}) failed to produce hyperpolarizing responses. Only strontium(Sr^{2+}) was able to substitute for calcium in evoking opening of potassium channels (a fuller account is given in Chapter III). However certain forms of stimulation by

Table 7. The effect of intracellular Ca^{2+} injection on the electrical properties of unfertilized hamster eggs.††

Cell No.	Recorded membrane potential (V) (mV)	Amplitude (ΔV) of Ca^{2+}-induced hyperpolarization (mV)	Recorded membrane potential (Fp) at the peak of the hyperpol. (mV)	Reversal potential (E_r) of the Ca^{2+}-hyper. (mV)	Input resistance prior to Ca^{2+} injection (MΩ)	Input Resistance at peak of hyperpol. (MΩ)	Duration of the Ca^{2+}-evoked hyperp. (sec)
1	-22	15	-37	-57	100	56	87
2	-24	18	-42	-93	130	96	response did not recover
3	-36	26	-58	-69	150	32	12
4	-66	0	-66	-66	110	16	—
5	-26	40	-66	-84	105	32	42
6	-32	23	-52	-72	225	97	14
7	-28	20	-48	-57	180	56	164
8	-35	25	-60	-77	78	35	30
9	-27	24	-51	-67	110	38	20
10	-17	27	-44	-71	72	36	26
11	-38	22	-60	-65	175	32	14
12	-44	22	-60	-63	175	24	19
13	-43	31	-74	-77	160	16	18
14	-32	22	-54	-63	160	48	10
15	-66	4	-70	-71	200	32	7
16	-16	25	-41	-87	68	44	71
17	-28	34	-62	-68	96	16	40
18	-30	24	-54	-63	99	27	30
19	-8	24	-32	-48	13	5	22
20	-23	27	-50	-68	120	48	22
21	-14	17	-31	-76	68	46	35
22	-38	24	-62	-70	110	44	12

23	-20	28	-48	-76	60	30	21
24	-20	25	-45	-57	52	17	29
25	-34	8	-42	-48	135	32	18
26	-42	31	-73	-109	116	62	30
27	-36	21	-57	-61	92	16	14
28	-33	25	-58	-66	80	20	12
29	-25	19	-44	-63	13	6	13
30	-30	26	-56	-76	46	20	11
31	-36	16	-52	-54	230	27	28
32	-43	20	-63	-73	420	89	18
33	-20	53	-73	-76	130	6	19
34	-20	20	-50	-52	38	15	21
35*	-16	27	-43	-85	42	25	32
36*	-19	40	-59	-81	275	100	29
37*	-18	25	-43	-52	95	25	38
38*	-18	27	-45	-64	100	50	45
39*	-26	40	-66	-75	125	25	17
40*	-28	26	-54	-67	100	36	22
41	-26	38	-64	-68	300	30	84
42	-10	30	-40	-70	60	32	32
43	-18	44	-62	-77	260	32	25
44	-20	45	-65	-73	375	25	44
45	-36	42	-78	-82	252	12	28
46	-31	33	-64	-74	460	85	22
47	-27	22	-49	-53	330	49	112
48*	-23	19	-42	-67	78	44	82
49*	-15	31	-46	-65	220	83	28
50*	-20	25	-45	-67	180	55	76
51*	-11	26	-37	-69	75	42	46
52*	-15	30	-45	-58	205	42	46

53*	-29	35	-64	-73	38	8	26
54*	-26	38	-65	-82	225	75	34
55*	-14	23	-37	-67	83	49	48
56*	-16	26	-42	-61	135	51	44
57*	-16	27	-43	-54	170	50	46
58*	-25	24	-49	-68	16	7	22
59*	-42	31	-49	-68	130	70	40
60*	-14	29	-43	-64	130	55	42
61*	-13	24	-37	-66	120	66	21
62*	-14	29	-43	-63	145	59	19
63*	-12	44	-56	-79	76	26	44
Mean$^{+}_{-}$SD	$-26^{+}_{-}12$mV	$27^{+}_{-}9$mV	$-53^{+}_{-}11$mV	$-69^{+}_{-}11$mV	$141^{+}_{-}94$MΩ	$40^{+}_{-}24$MΩ	$35^{+}_{-}27$ sec (n=61)

† All values quoted (except for cell 2) refer to mean values.

* The membrane potential and the input resistance were monitored via the Dagan amplifier in results indicated by the asterisk.

The duration of the calcium current pulse was either 0.5 or 1 sec.

hyperpolarizing pulses passed through either the recording or the injecting electrode were found capable of eliciting responses similar to those produced by injecting Ca^{2+} (see Sections 6, 7 & 8). For any constant amplitude of the ionophoretic current pulse, the amplitude and duration of the resulting hyperpolarizing responses were graded according to the duration of the pulse. This gradation, typical examples of which are shown in Fig 7, suggests a relation between the amount of injected calcium ions and the extent of the subsequent hyperpolarization. (It is assumed that a proportionality exists between the duration of a given pulse and the quantity of calcium ions liberated from the microelectrode tip.)

Plots of half-maximal duration and peak amplitude of the hyperpolarizing responses against calcium pulse duration for two different eggs are given in Fig.8. For calcium pulses >300ms the half maximal duration of the hyperpolarization showed an apparent wide variability from cell to cell (see also similar data on mouse eggs). It is likely that the major part of this variability reflects current passage limitations of different calcium microelectrodes rather than wide differences in sensitivity among cells to changes in intracellular levels of ionized calcium.

To determine whether the calcium evoked hyperpolarization was accompanied by any changes in the cell membrane conductance, constant hyperpolarizing

Figure 7

Effect of the duration of the current pulse passed through a calcium pipette on the size and duration of the subsequent membrane hyperpolarization in hamster eggs. The pulse durations from top to bottom were 1.00, 0.50, 0.25, 0.10 and 0.05 seconds. The value of the membrane potential prior to each Ca^{2+} injection is indicated at the left of each trace.

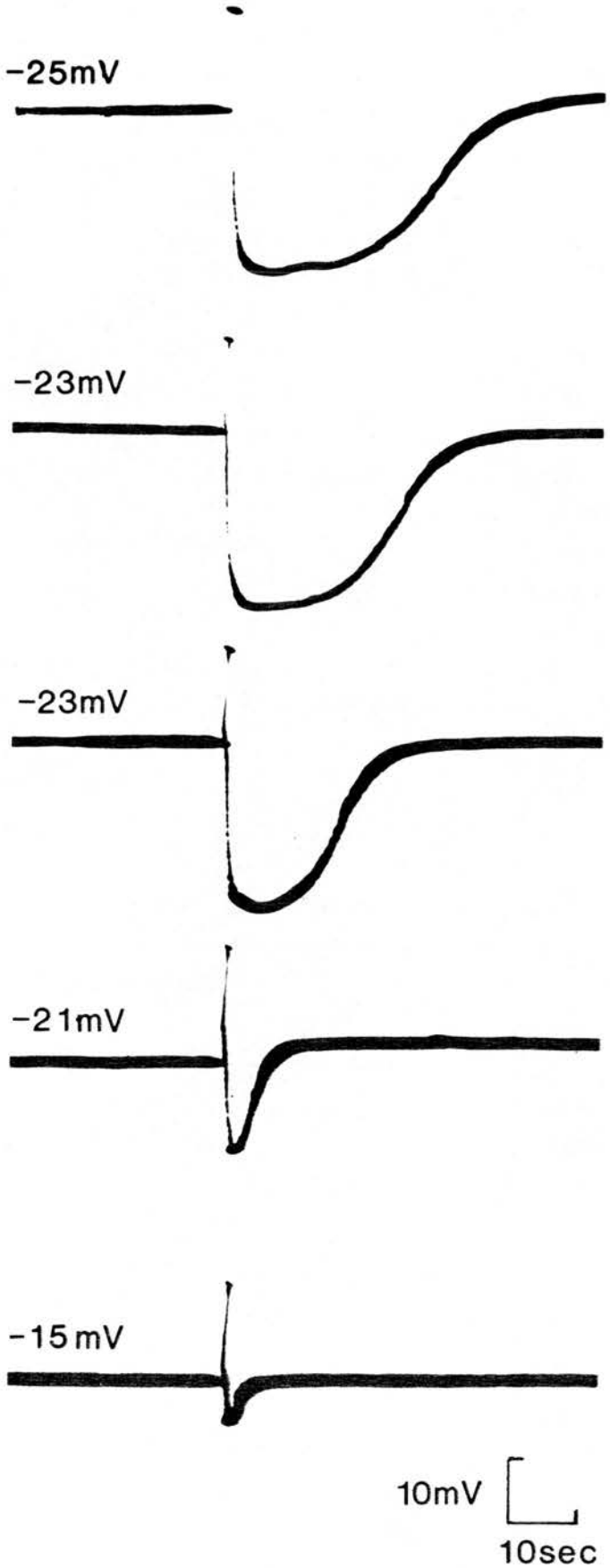
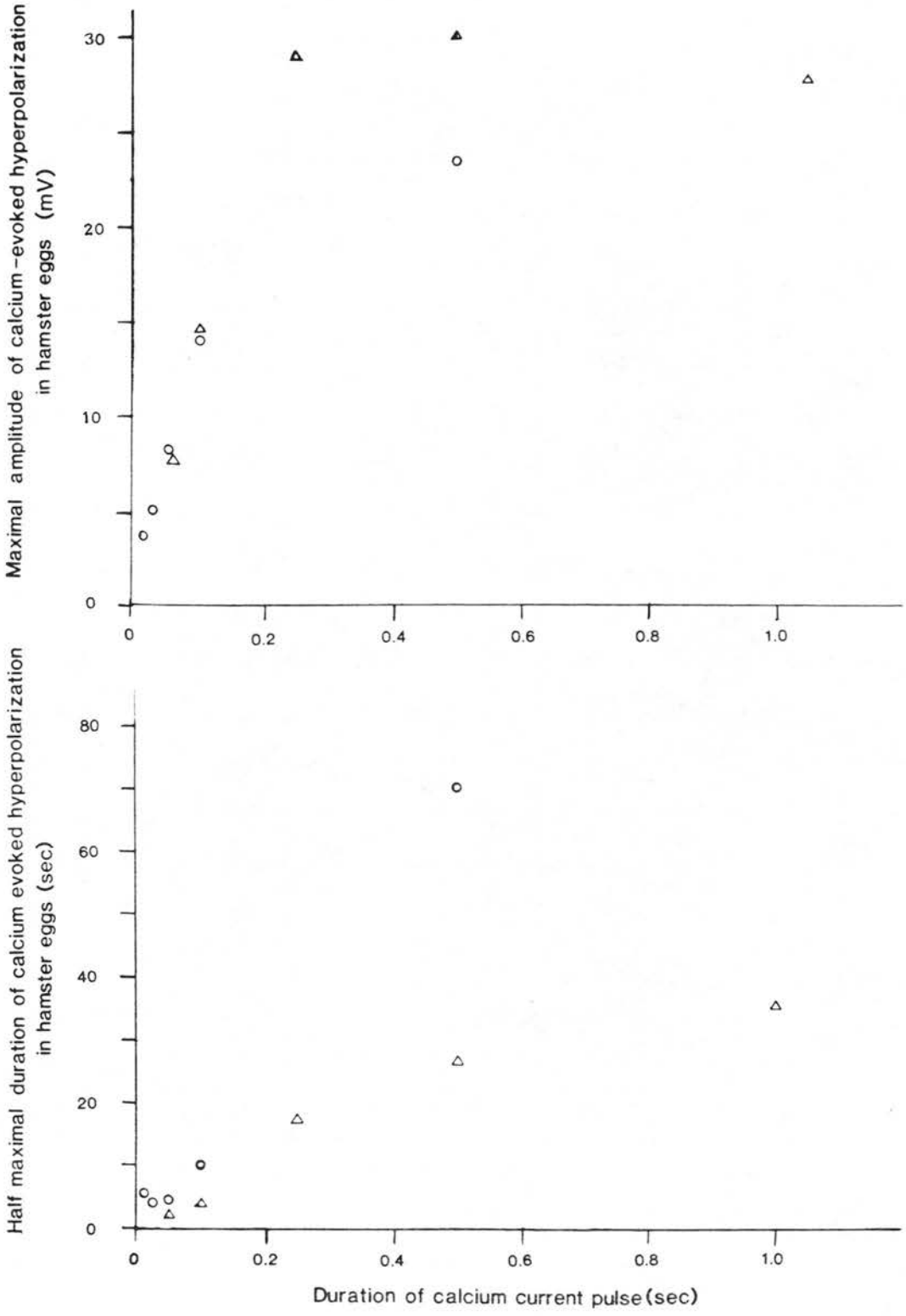

-25mV
-23mV
-23mV
-21mV
-15mV
10mV
10sec

Figure 8

Graphs showing the gradation in peak amplitude and half-maximal duration of the calcium evoked hyperpolarization in two different eggs as functions of the calcium current pulse duration. In both top and bottom graphs the open circles indicate measurements of maximal amplitude and half-maximal duration taken from the same egg whereas open triangles refer to measurements taken from another egg.


Maximal amplitude of calcium-evoked hyperpolarization in hamster eggs (mV)
30
20
10
0
0
0.2
0.4
0.6
0.8
1.0
Half maximal duration of calcium evoked hyperpolarization in hamster eggs (sec)
80
60
40
20
0
0
0.2
0.4
0.6
0.8
1.0
Duration of calcium current pulse (sec)

current pulses were passed through the recording microelectrode. Hyperpolarizing current pulses applied were such that the amplitude of the resulting electrotonic potentials lay in the linear region of the current voltage relation which in the hamster egg extends from 0 to -150mV. Representative records of simultaneous monitoring of membrane potential and membrane input conductance during a calcium injection are shown in Fig.9. The experimental records of Fig 9 show that a marked reduction in the cell input resistance occured during the calcium-evoked hyperpolarization as judged by the large reduction in the size of the electrotonic potentials (shown as downward deflections on the voltage trace) which are proportional to membrane resistance.

Section 4 : Calculation of the reversal potential(E_r)

The reversal potential of the calcium-evoked hyperpolarization was estimated according to the analysis of Trautwein and Dudel (1958). The end result of such an analysis (given in Appendix 2) is the following equation:

$E_r = (P/P-p)\Delta V + V$..... (3) where

E_r is the reversal potential of the hyperpolarization

V is the measured membrane potential

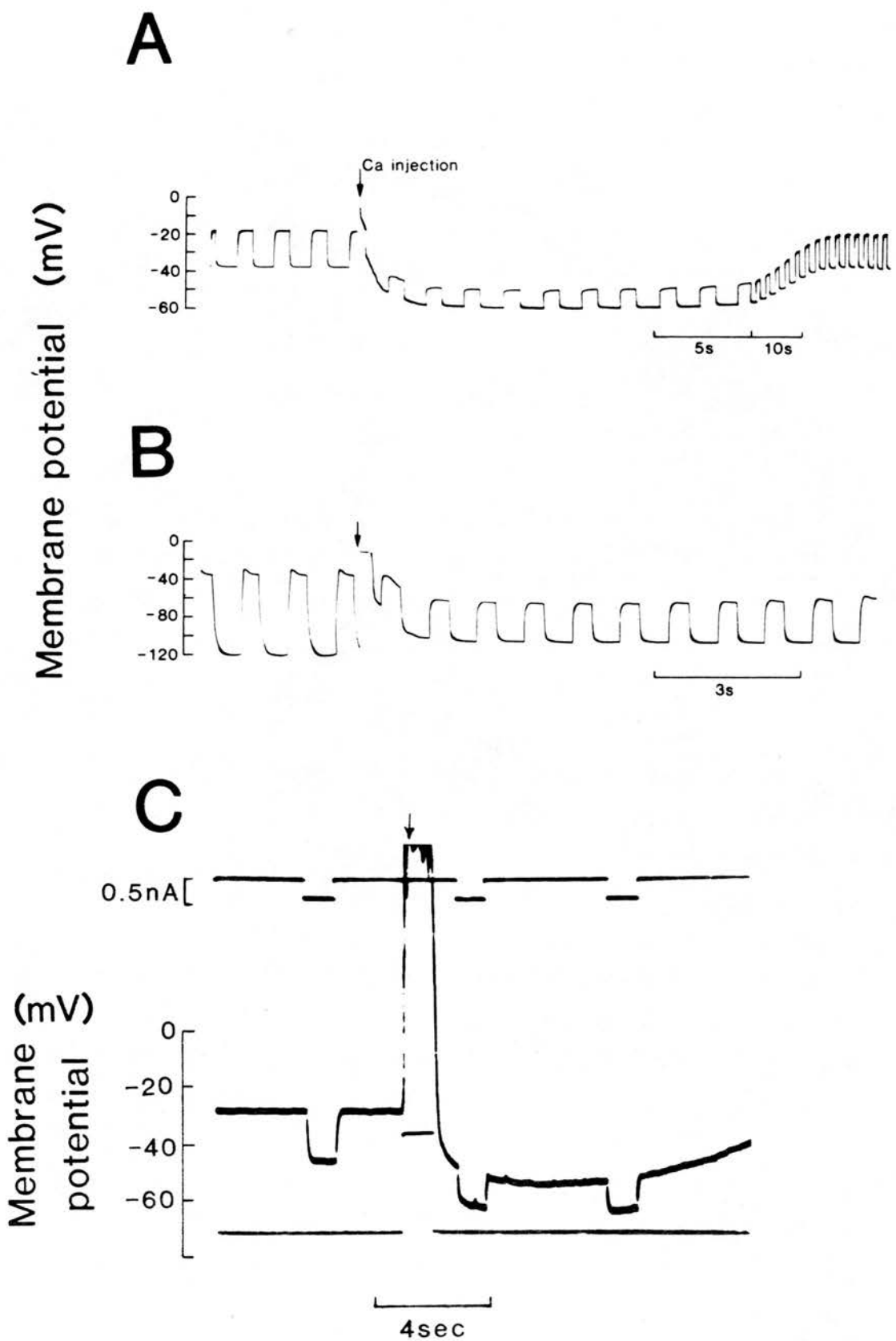
ΔV is the measured amplitude of the hyperpolarization

P is the size of the electrotonic potential prior to calcium stimulation

p is the size of the electrotonic potential at the peak of the hyperpolarizing response.

Figure 9

Effect of calcium ions ionophoretically injected into hamster eggs on membrane potential and resistance. Arrows in A and B indicate the time at which calcium was ionophoretically injected into eggs. Traces A and B show results of experiments on two different eggs. The hyperpolarizing electrotonic potentials were produced by constant current pulses (not shown)passed through the recording microelectrodes. C is an oscilloscope picture of a hyperpolarization evoked by injecting a 5nC pulse (lowest trace) through the pipette into an egg. The voltage deflections are electrotonic potentials evoked in response to the application of constant hyperpolarizing current pulses (top trace) through the recording microelectrode. In all three experiments injection of Ca^{2+} into the eggs caused a substantial membrane hyperpolarization as judged by the large reduction in the amplitude of the electrotonic potentials.


A
Ca injection
0
-20
-40
-60
5s
10s
Membrane potential (mV)
B
0
-40
-80
-120
3s
C
0.5nA
(mV)
Membrane potential
0
-20
-40
-60
4sec

Equation (3) is valid only if the the resting conductance is not dependent on the applied current and hence on the value of the membrane potential . In other words it is only applicable within the linear region of the current-voltage relation . Therefore the strength of the current pulses used were such that the resulting electrotonic potentials fell within the linear region of the current-voltage relation as already mentioned in the preceding section.

To illustrate this method of analysis consider the example shown in the top trace of Fig.9. The recorded values of V , ΔV , P , p were -19mV , -30mV , -20mV and -10mV respectively, which yield E_r=-79mV. In 63 cells the above method of analysis produced an estimated mean value for the reversal potential of -69 $\pm$ 11mV (see Table 7). This value is lower than the value of the reversal potential (-80mV) reported by Miyazaki and Igusa (1982). The mean values of the reversal potential presented in Table 7 (section 4 in Part II of this chapter) were probably underestimated since an injection-induced membrane beakdown (described in Chapter III) might have occured during calcium-evoked responses.

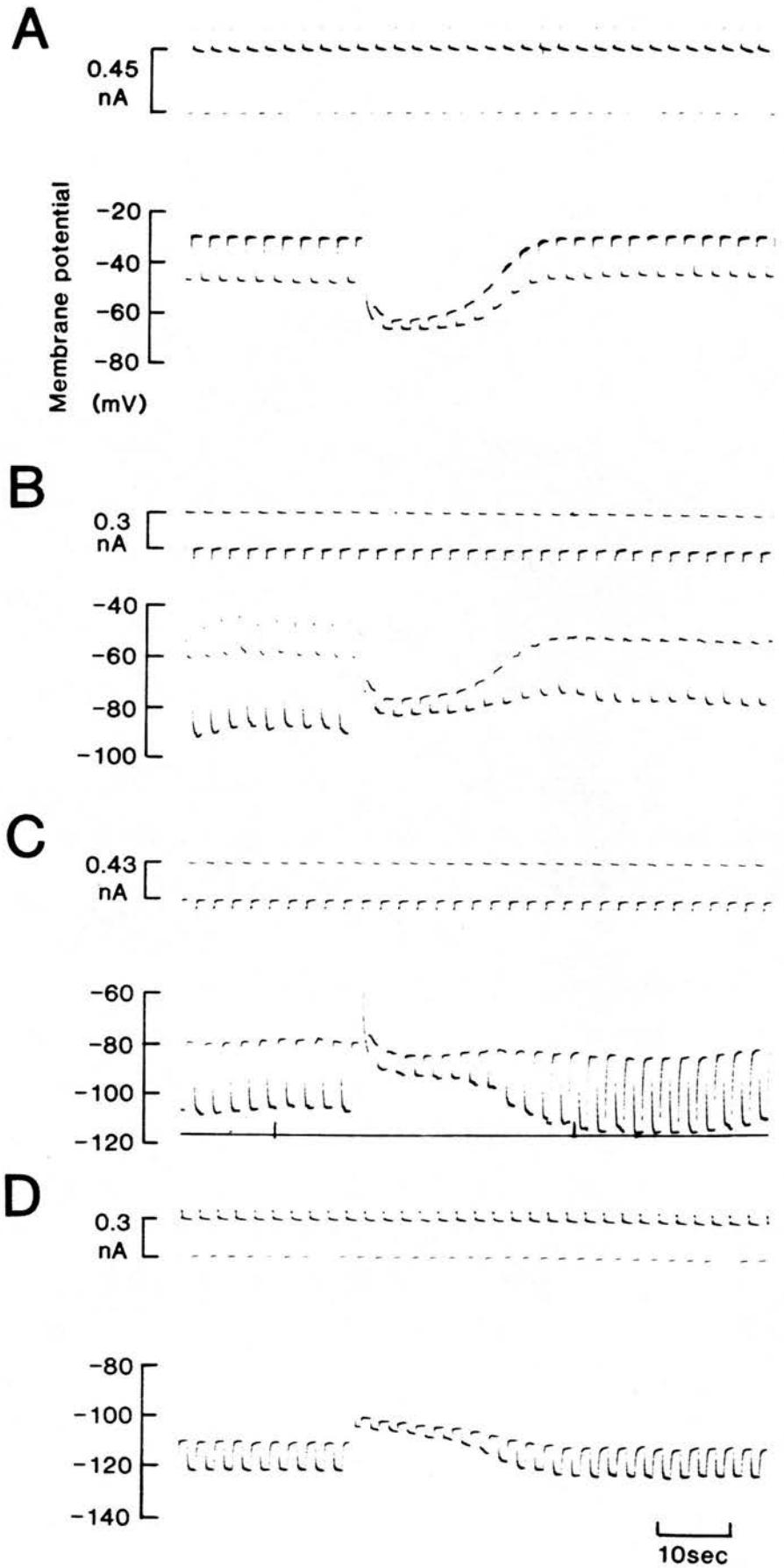
Section 5 :Reversal of the polarity of the Ca^{2+}-evoked response

In agreement with the findings of Miyazaki and Igusa(1982) a clear reversal of the calcium-evoked response (n=3) from a hyperpolarization to a depolarization could be achieved when the membrane

potential was shifted to values beyond the reversal potential of the response by the application of constant (dc) current through the recording microelectrode. Fig. 10 shows that the amplitude of the Ca^{2+}-evoked potential change is symmetrical about the reversal potential (-80mV) which argues against a possible involvement of an electrogenic pump in the generation of the response.

Figure 10

The effect of membrane potential on the polarity of the calcium-evoked response. A and B show that at membrane potentials more positive than -80mV calcium injections evoked a hyperpolarization. C shows that when the membrane potential was current-clamped at a value close to the reversal potential of the response, hardly any change in potential occurs following a pulse of calcium ions into the egg. Notice however the prominent fall of membrane resistance indicative of channel opening. D shows that shifting the membrane potential to values more negative than -80mV changed the sign of the calcium-evoked response to a depolarization which was again accompanied by a marked reduction in membrane input resistance.


A
0.45
nA
Membrane potential
-20
-40
-60
-80
(mV)
B
0.3
nA
-40
-60
-80
-100
C
0.43
nA
-60
-80
-100
-120
D
0.3
nA
-80
-100
-120
-140
10sec

Section 6 :Ionic basis of the calcium-evoked hyperpolarization

Reduction of the external chloride concentrations from 135 to 12mM did not cause any change in the value of the reversal potential of the calcium evoked-hyperpolarization. (E_r = -62±3mV in 135mM $[Cl]_o$ and -64±14mV in 12mM $[Cl]_o$, n=4 exps). This indicates that the calcium-induced hyperpolarization is not brought about by the opening of chloride channels activated by a rise in the level of ionized free calcium. A typical record of an experiment carried out in a chloride reduced solution is shown in Fig.11.

By contrast a linear relation between the reversal potential of the responses and the external potassium concentration was found. This is illustrated in Fig. 12 which shows the effect of different external concentrations of potassium ions on the reversal potential (open circles). The mean slope of the relation between the reversal potential and $\log_{10}[K]_o$ for the results presented in Fig.12 was found to be 50mV which is in good agreement with the value of 58mV predicted by the Nernst equation.

A representative example of an experiment where calcium-evoked responses were obtained in solutions containing 1 and 125mM potassium is shown in Fig.13. The figure illustrates that the direction of the potential change reversed when the external concentration of potassium was changed from 1 to 125mM. The calculated reversal potential for $[K^+]_o$ = 1mM was

Figure 11

The effect of reducing the concentration of external chloride on the calcium-evoked hyperpolarizations. A shows hyperpolarizations obtained in normal solution whereas B and C shows calcium-evoked hyperpolarizations in a solution with chloride concentration of 12mM. The responses shown in D were obtained after the chloride concentration was restored to its normal level (130mM). Throughout the experiment identical calcium current pulses were used to elicit electrotonic potentials.

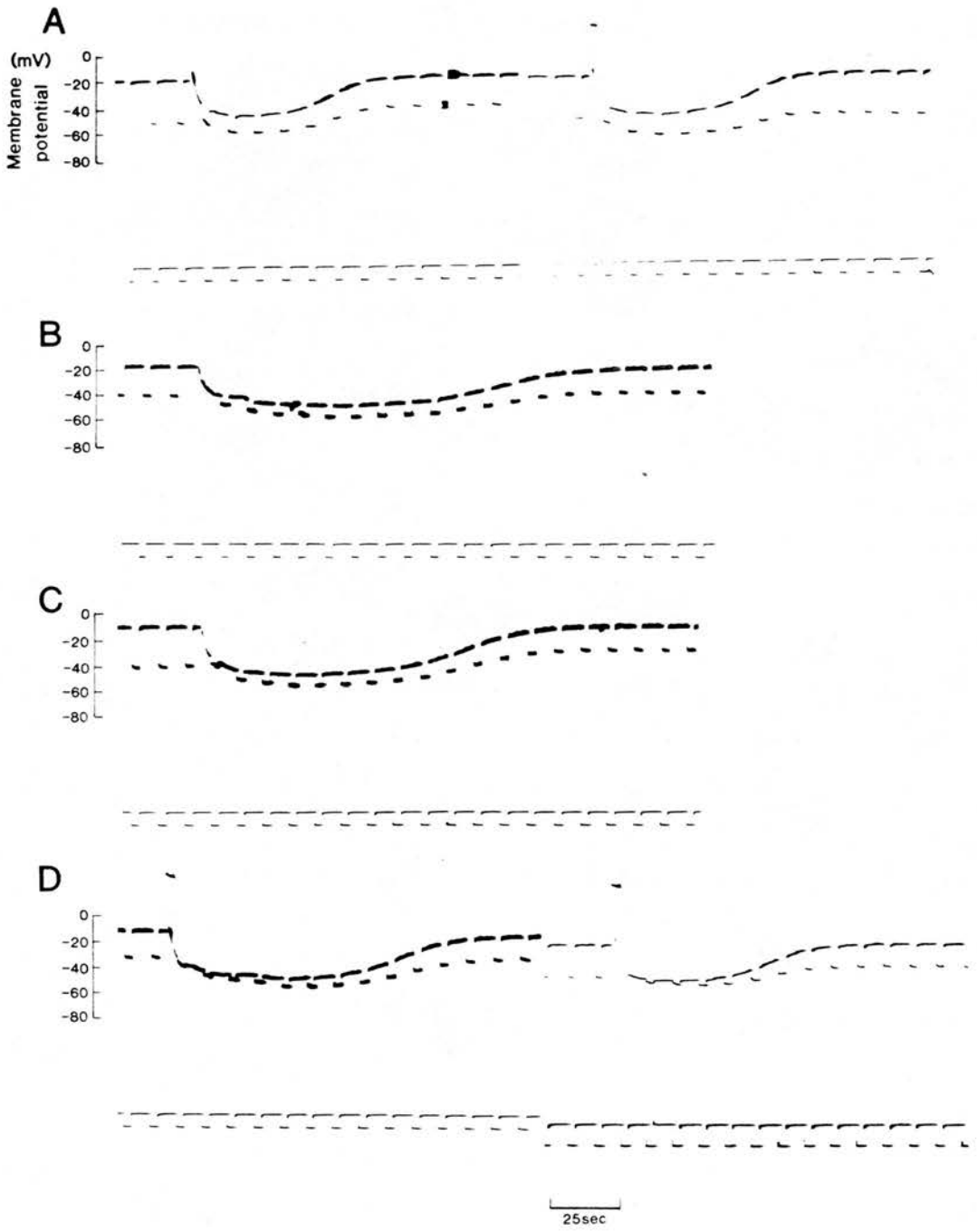

A
(mV)
Membrane potential
0
-20
-40
-60
-80
B
C
D
25sec

Figure 12

Effect of the extracellular concentration of potassium on the resting potential (filled circles) and the estimated reversal potential (open circles) in the same cell during the response to injected calcium.

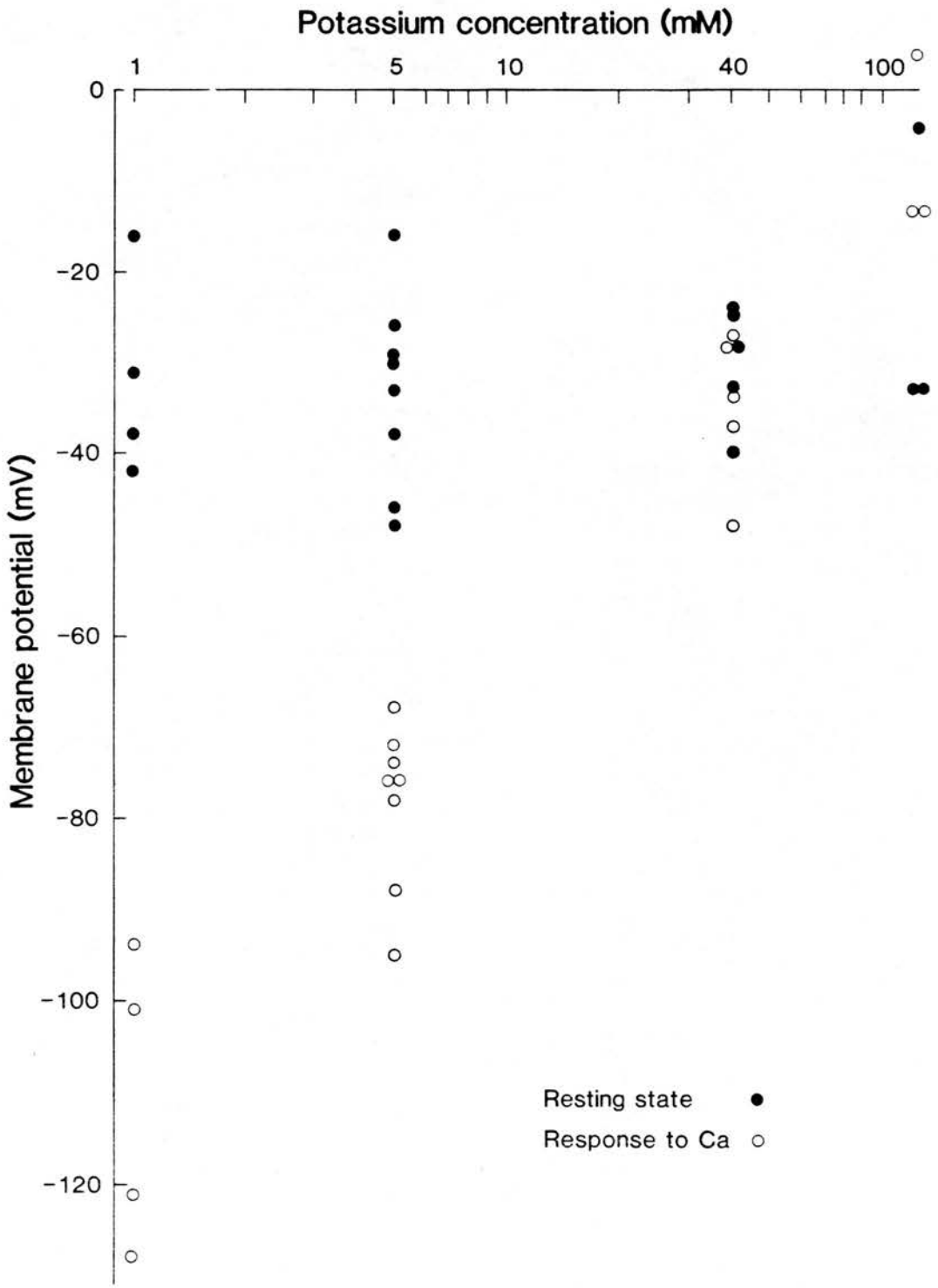
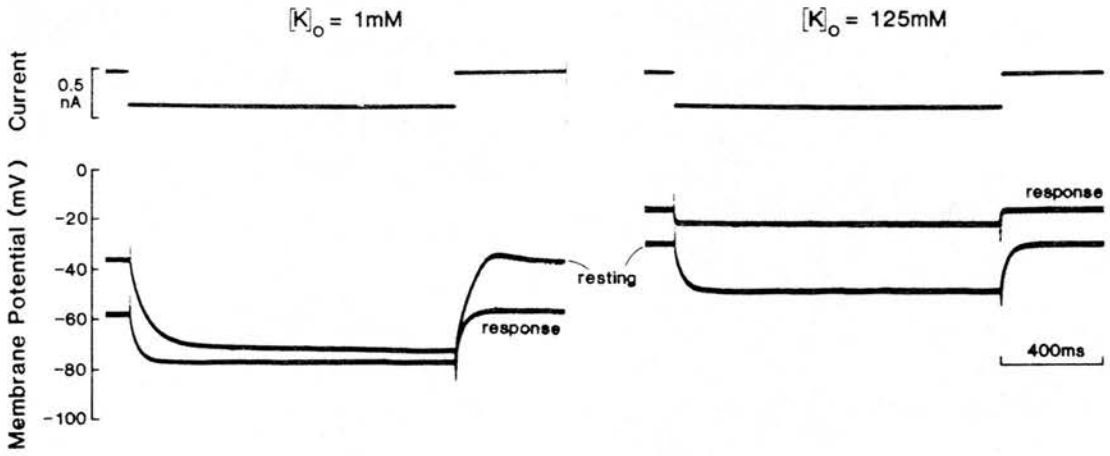

Potassium concentration (mM)
1
5
10
40
100
0
-20
-40
-60
-80
-100
-120
Membrane potential (mV)
Resting state
Response to Ca

Figure 13

Effect of the extracellular potassium concentration on the electrical changes in response to calcium injection into a hamster egg. The oscilloscope picture on the left shows that in the presence of $[K]_o$=1mM ionophoretically injected Ca^{2+} caused a membrane hyperpolarization as indicated by the difference between the voltage baselines. However , when the $[K]_o$=125mM the effect of a Ca^{2+} pulse was a membrane depolarization . The top trace of both pictures shows a constant hyperpolarizing current pulse applied through the recording microelecrode for the determination of the cell input conductance. Notice that a large increase in input conductance occurs during the response irrespective of $[K]_o$


[K]o = 1mM
[K]o = 125mM
Current
0.5 nA
Membrane Potential (mV)
0
-20
-40
-60
-80
-100
resting
response
response
400ms

-98mV and when $[K^+]_o$ was elevated to 125mM E_r became -13mV. The expected shift in the value of the reversal potential, assuming perfect selectivity towards K^+ would be 122mV. However, the experimentally determined shift in this particular case amounted to 85mV. Nevertheless, it still strongly suggests a conductance change primarily selective for K^+.

Section 7 :Anode-break evoked responses

Trains of hyperpolarizing current pulses of short duration (300-500ms) at a frequency of 1-2Hz and of sufficient strength (0.25-0.5nA) to elicit anode-break responses (calcium action potentials) were occasionally found to be effective in eliciting hyperpolarizing responses. Examples of responses obtained by this kind of stimulation are shown in Fig.14. The estimated reversal potentials for these responses were -90mV and -80mV (Fig.14 A and B) respectively, thus strongly suggesting a calcium mediated potassium efflux as the cause for the potential response.

The most likely explanation of this phenomenon is that calcium movement into the egg during a train of anode-break responses raises the activity of cytosolic free calcium to a level sufficient to effect opening of K^+-channels. This kind of summation effect has also been observed in molluscan neurones (Connor & Stevens, 1971; Brodwick & Junge, 1972; Moreton, 1972; Meech, 1974b)

In some experiments, such as the one shown in Fig.15, it was found that the anode break response

Figure 14

Examples of hyperpolarizations evoked by repetitive and constant hyperpolarizing current pulses. Notice that in both A and B the value of the membrane voltage at the peak of the electrotonic potentials (brief downward deflections) elicited in response to the application of constant hyperpolarizing current pulses (not shown) is greater than -80mV. The upstroke phase in the electrotonic potentials is characterized by a slight overshoot of the voltage baseline (top continuous black line shown in the records of A and B) due to the generation of anode break responses. Once the membrane voltage is shifted to -80mV calcium channels are no longer inactivated with the result that at the end of the pulse a calcium action potential is initiated (anode-break response ; Okamoto, Takahashi & Yamashita,1977 ; Miyazaki & Igusa,1982)

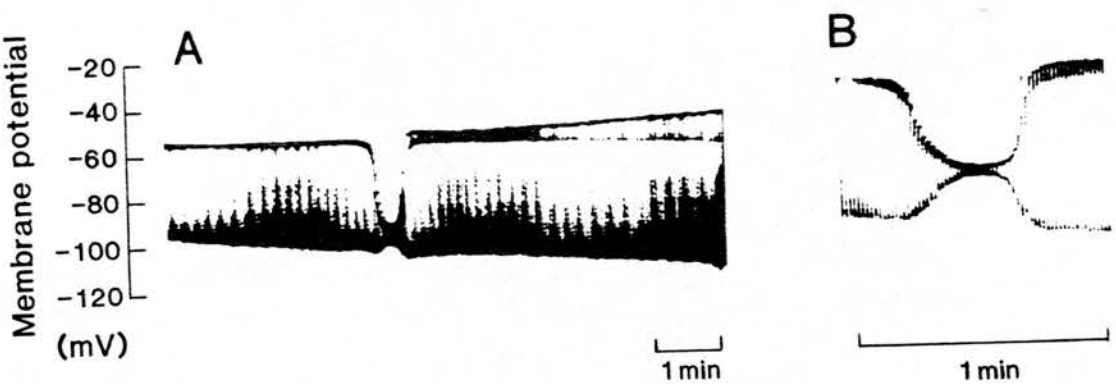
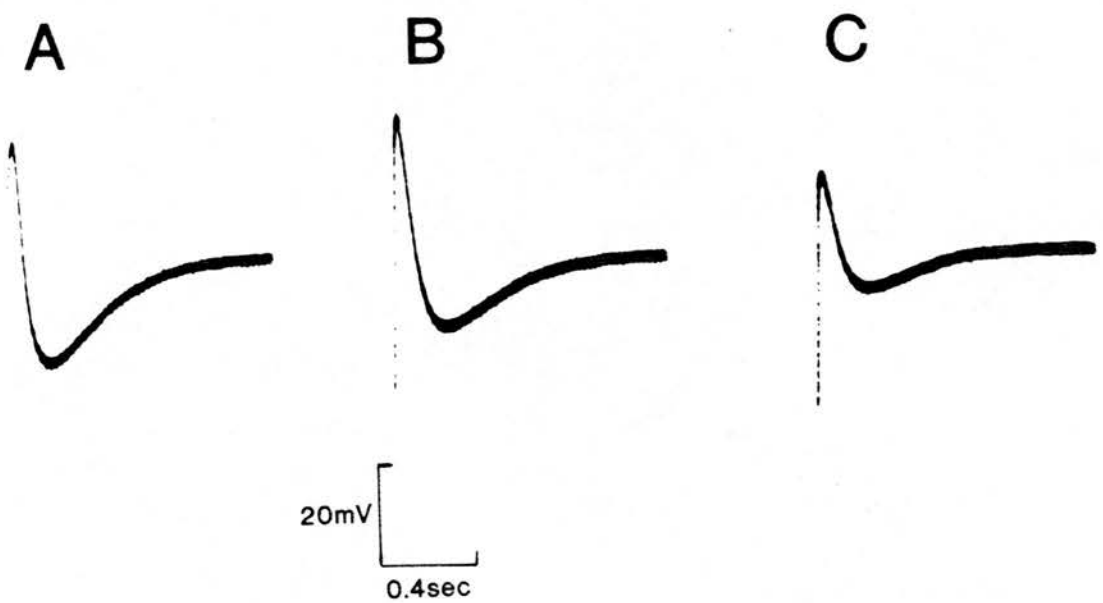

A
B
Membrane potential
-20
-40
-60
-80
-100
-120
(mV)
1 min
1 min

Figure 15

The effect of increasing the external potassium concentration on the amplitude of the after-hyperpolarization that occasionally follows anode break responses. A progressive reduction in the size of the after-hyperpolarization is observed as the potassium concentration is raised from 5mM(A) to 15mM(B) and finally to 25mM(C).


A
B
C
20mV
0.4sec

was followed by a prominent after-hyperpolarization. As shown in Fig.15 the size of the after-hyperpolarization was dependent on the external potassium concentration, enhanced at 5mM external potassium and markedly reduced at relatively high external potassium (25mM). Restoration of the normal 5mM potassium concentration produced a partial recovery of the after-hyperpolarization. In the example shown in Fig. 15, the observed shift in the minimum value of the membrane potential during the anode-break response was 5mV when the $[K^+]_o$ was raised from 5 to 15mM and 6mV when $[K^+]_o$ was elevated from 15 to 25mM . The corresponding shifts predicted by the Nernst equation are 27 and 12mV respectively . The apparent disparity between the observed shifts of potential and those predicted by the Nernst equation might be due to the presence of a substantial leakage current and/or to persistent calcium movement occurring during the after-potential. Okamoto, Takahashi and Yamashita (1977) have observed similar results in mouse eggs where they found that the minimum value of the membrane potential during an anode-break response changed by about 15mV when $[K]_o$ was raised from 6 to 30mM and by about 27mV when $[K]_o$ was raised from 30 to 133mM. Their interpretation of the results was that the membrane permeability to K^+ was raised during the hyperpolarizing potential and declined during the tail of the anode-break response.

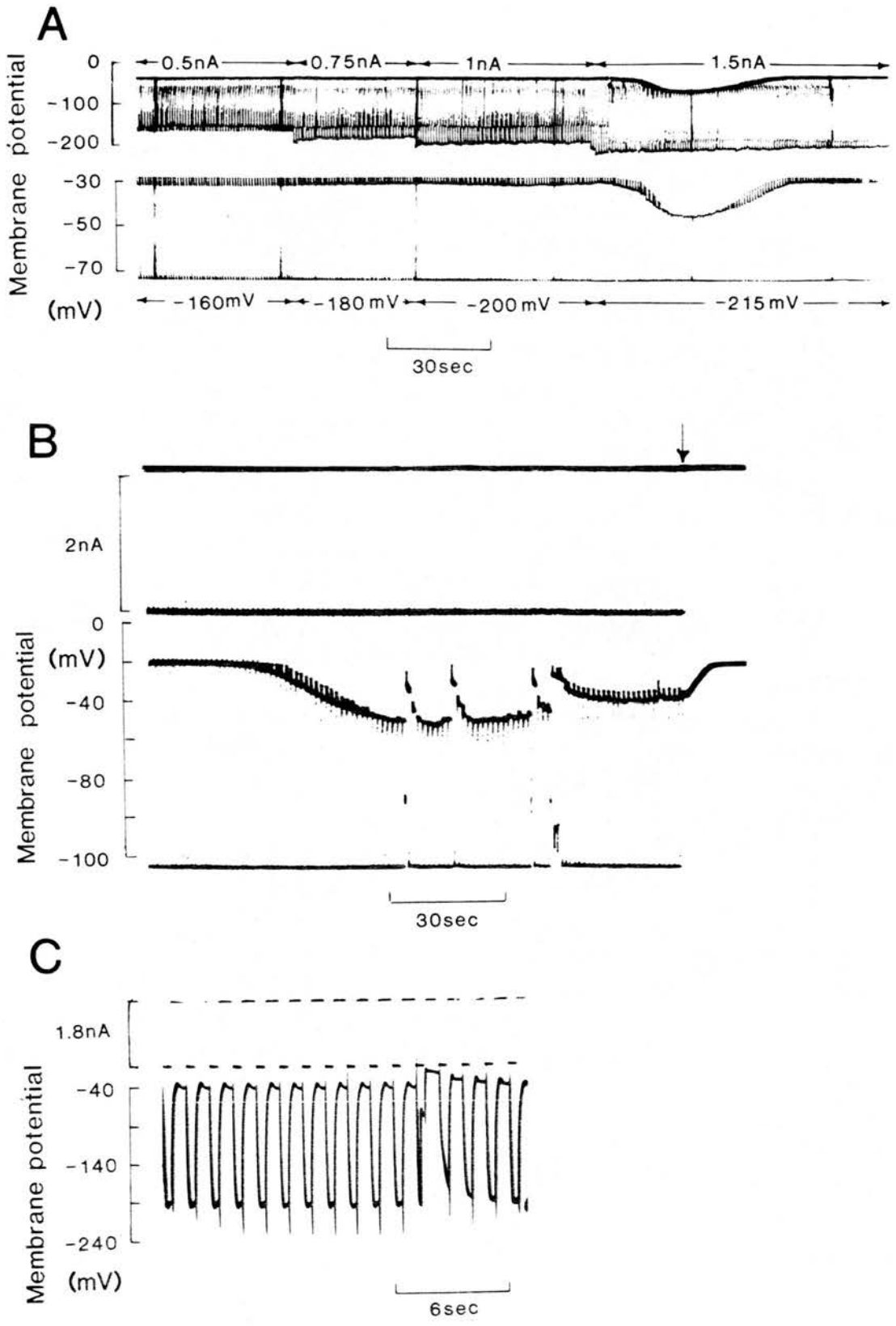
Section 8 : Responses to hyperpolarizing current pulses per se

Repetitive application of o.4sec hyperpolarizing current pulses (at a frequency of 2-3Hz) of strength > 1.5nA could evoke hyperpolarizations (n=16) as shown in the traces of Fig. 16. The records of Fig.16 show that the membrane was hyperpolarized to values close to or exceeding -200mV during application of repetitive current pulses of > 1.5nA. As previously mentioned (see Section 1 in Part I of this chapter) the current-voltage relation becomes highly non-linear at membrane voltages exceeding -150mV. Moreover at such negative voltages the membrane voltage exhibited marked fluctuations associated with a reduction in the input resistance (see Fig.16 B & C), which are probably indicative of temporary membrane breakdown. It is therefore plausible that hyperpolarizations caused by hyperpolarizing current pulses such that the membrane voltage was displaced beyond -150mV were predominantly caused by calcium entry through low resistance pathways due to temporary membrane breakdown. Hodgkin (1947) observed that non-medullated axons when subjected to excessive anodic polarizations also suffered a gradual loss in resistance. Del Castillo and Katz (1955) reported a similar phenomenon for the nodes of Ranvier of frog sciatic nerves. They found that "anodic breakdown " as they called it occurred when the node was polarized to voltages lying in the range of -150 to -400mV. Coster (1965) working on the membranes of the

Figure 16

The induction of hyperpolarizing responses in response to hyperpolarizing current pulses of moderate strength (>1.5nA). The top and bottom traces of A show the same voltage (pen) recording taken at different gains. The downward deflections indicate electrotonic potentials elicited in response to hyperpolarizing current pulses , the strength of which remained constant for the time periods indicated . The values of the current pulses and the corresponding values of the membrane potential attained at the peak of the electrotonic response are also shown. Note that a hyperpolarization was elicited only after the value of the current pulse was raised to 1.5nA . This current caused a displacement of the membrane potential to -215mV. B is another example of a hyperpolarizing response evoked by current pulses (upper trace) which displaced the membrane potential (lower trace) beyond the linear region of the current-voltage relation (-150 to +50mV). Notice the abrupt fluctuations in the membrane voltage once the response is maximal. At the arrow the current pulses were switced off and the membrane potential returned to its original value. C shows electrotonic potentials (lower trace) obtained during the peak of a hyperpolarization caused by constant current pulses (upper trace). Notice the spontaneous fluctuations in the membrane potential. Unfortunately in the lower portion of figures A and B the bottom edge of the trace is straight due to the

pen reaching the physical end of its travel.


A
0
-100
-200
Membrane potential
0.5nA
0.75nA
1nA
1.5nA
-30
-50
-70
(mV)
-160mV
-180 mV
-200 mV
-215 mV
30sec
B
2nA
0
(mV)
-40
-80
-100
Membrane potential
30sec
C
1.8nA
-40
-140
-240
(mV)
Membrane potential
6sec

giant algae, Chara and Nitella also produced evidence of electrical membrane breakdown, which he called "punch- through" in analogy to a similar kind of phenomenon observed in semiconductors. The value obtained by Coster for the membrane voltage at which "punch-through" occured about -300mV. The development of a high conductance state induced by hyperpolarization of the cell membrane beyond -100mV has also been reported by Marmor (1971) in the giant neurone (G cell) of Anisodoris. The critical potential beyond which the high conductance state developed was found to vary with temperature being -110mV at 16°C and -130mV at 0°C. The critical potential was not found to depend on the presence of either $[K^+]_o$, $[Na^+]_o$ or $[Cl^-]_o$ whereas it was markedly affected by changes in $[Ca^{2+}]_o$. Lowering external calcium caused it to appear at less hyperpolarized potentials in both the cold(0°C) and the warm(16°C). Marmor also observed an inward-going rectification dependent on $[K^+]_o$ in the same preparation but concluded that it could not account for the development of the high conductance state since the latter exhibited no specificity towards K^+. Marmor suggested that certain fixed charge membrane models such as the ones proposed by Mauro(1962) and by Coster(1965) could account for the generation of the high conductance state at high negative potentials.

That these responses result from opening of Ca^{2+}-activated K^+-channels is supported by the finding that the sign of the potential response could be reversed

from a hyperpolarizing one to a depolarizing one when the the external potassium concentration was raised from 1 or 5mM to 125mM. This is illustrated in Fig.17.

The effectiveness of membrane breakdown was greatly facilitated by larger hyperpolarizing current pulses and inward current pulses of > 10nA almost always produced hyperpolarizations. Equally large depolarizing current pulses failed to elicit responses. Representative examples of such "excessive" current-induced responses are shown in Fig.18a. Responses induced by large hyperpolarizing current pulses were also observed in the presence of external lanthanum (0.1-0.5mM) a potent blocker of voltage sensitive calcium channels in hamster eggs (Georgiou, Bountra, Bland & House) . This is illustrated in Fig.18b. The application of large hyperpolarizing current pulses displaced the membrane potential to values exceeding -500mV. Coster and Zimmermann (1975) quote a value of -600mV for the voltage beyond which breakdown of the membrane dielectric occured.

Section 9 :Calcium injections in mouse eggs

Injections of calcium ions into mouse eggs evoked hyperpolarizing responses similar to those observed in hamster eggs but in general of lesser amplitude ($\Delta V = 6 \pm 4mV$, n=17). The largest response obtained in these eggs during the whole course of these injection experiments was 12mV. As in the case of hamster eggs

Figure 17

Responses of an egg to trains of hyperpolarizing current pulses (> 1.5nA) passed through the recording microelectrode while bathed in different potassium concentrations. The times after impalement at which the current pulses were delivered are given above each extract from the continuous record of the membrane potential. The horizontal bars underneath each trace were generated by the pen hitting the physical end of its travel in the pen-recorder. Notice the change in the polarity of the response from a hyperpolarization to a depolarization as the external potassium concentration is raised from 5 to 125mM.

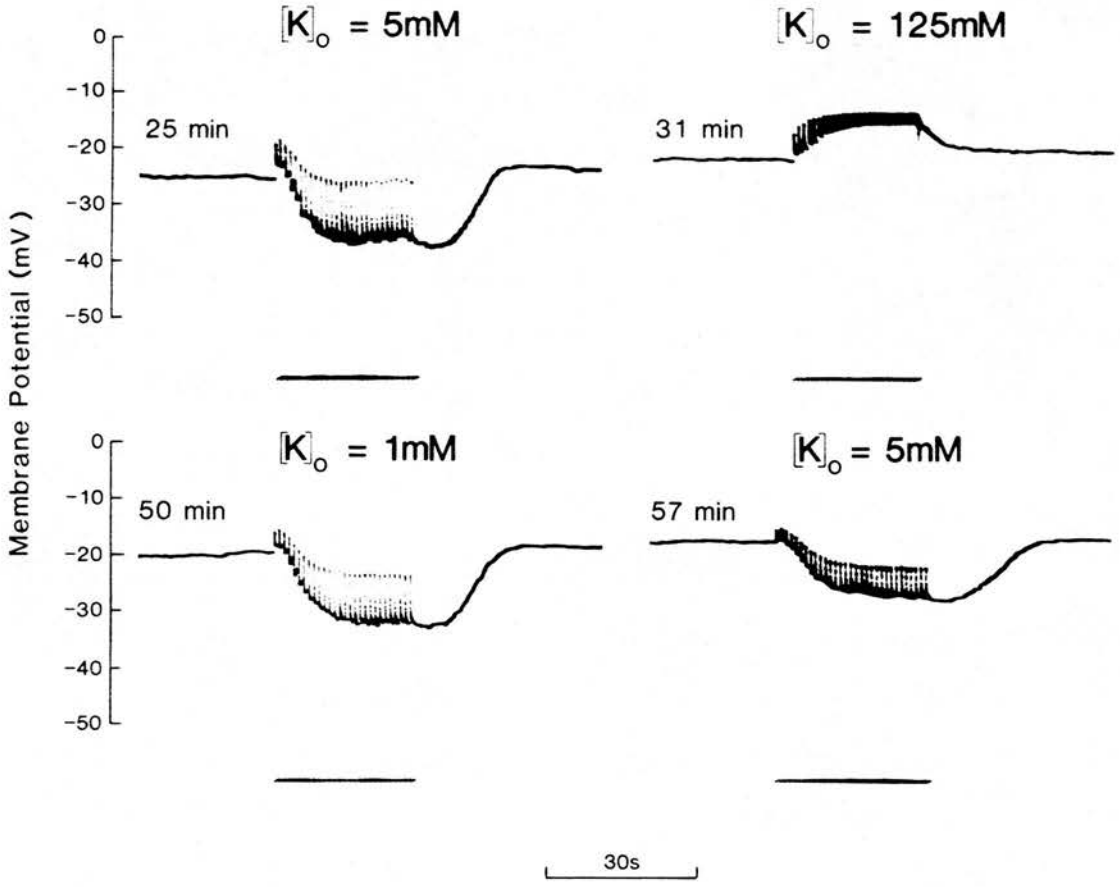

[K]o = 5mM
[K]o = 125mM
[K]o = 1mM
[K]o = 5mM
25 min
31 min
50 min
57 min
0
-10
-20
-30
-40
-50
Membrane Potential (mV)
30s

Figure 18a

The "breakdown" effect of the hamster egg plasma membrane by large hyperpolarizing current pulses. A, B and C are recordings taken from the same egg. A and B show that depolarizing current pulses of 10nA (1sec duration) failed to elicit any active electrical response whereas hyperpolarizing current pulses of the same strength shown in C evoked a hyperpolarizing response associated with a marked reduction in input resistance very reminiscent of hyperpolarizations evoked by calcium injections. Downward deflections (electrotonic potentials) on A, B and C were produced by constant 0.3nA (1sec duration) hyperpolarizing current pulses. The brackets labelled a, b and c show the period of electrical stimulation by large current pulses. D shows that a single 10nA (1sec in duration) hyperpolarizing current pulse was sufficient to evoke an active electrical response in another hamster egg. E shows that during the application of large hyperpolarizing current pulses the membrane voltage was displaced to values of greater or equal to -500mV and in addition it displaed marked and erratic fluctuations indicative of membrane breakdown.

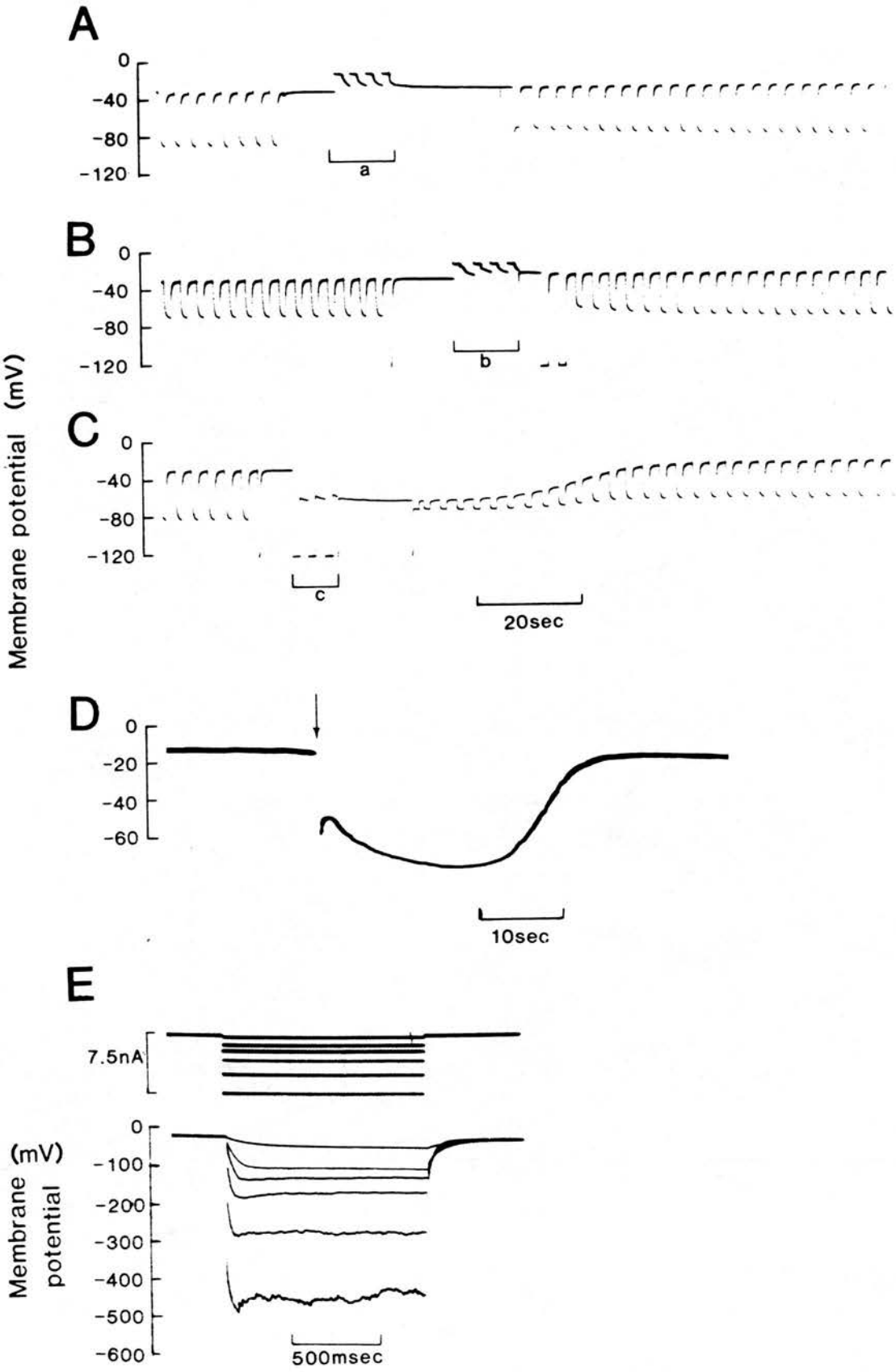
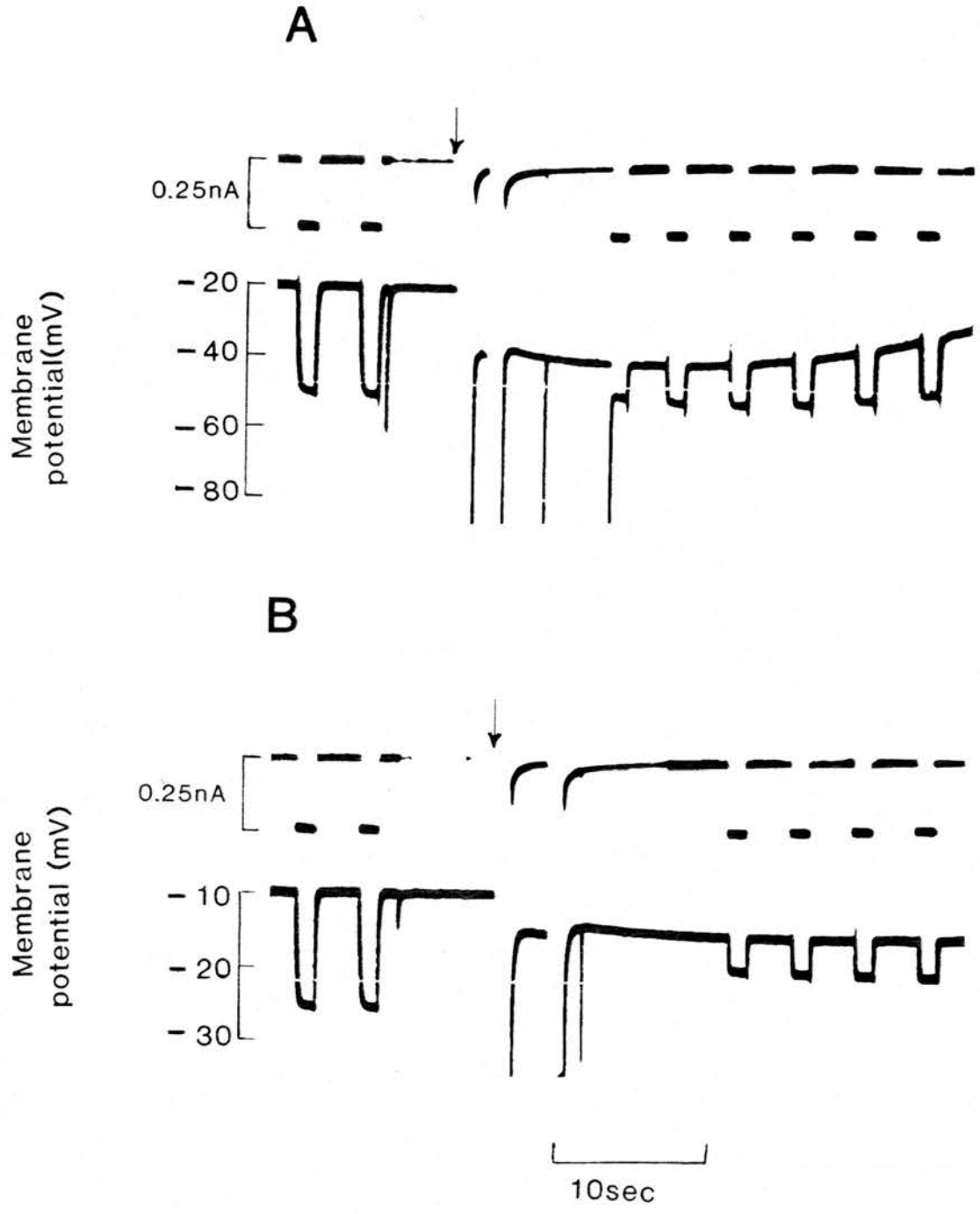

A
0
-40
-80
-120
a
B
0
-40
-80
-120
b
C
0
-40
-80
-120
c
20sec
Membrane potential (mV)
D
0
-20
-40
-60
10sec
E
7.5nA
0
-100
-200
-300
-400
-500
-600
(mV)
Membrane potential
500msec

Figure 18b

Hyperpolarizations evoked in response to large hyperpolarizing current pulses in the presence of external lanthanum. In both the oscilloscope pictures shown in A and B application of large hyperpolarizing current pulses (30 and 50nA respectively; time of application indicated by the arrows above the current traces) evoked membrane hyperpolarizations followed by a prominent reduction in the size of the electrotonic potentials (voltage deflections) which is indicative of a drop in input resistance.


A
0.25nA
-20
-40
-60
-80
Membrane potential(mV)
B
0.25nA
-10
-20
-30
Membrane potential (mV)
10sec

the amplitude and duration of the calcium-induced hyperpolarization were graded according to the quantity of charge ejected from the calcium-pipette. Representative records of such relations are illustrated in Fig.19.

Graphs of the half-maximal duration and maximal amplitude against the duration of the Ca^{2+} pulse for the responses illustrated in Fig.19 are shown in Fig.20 It appears from these plots that both half-maximal duration and maximal amplitude tended to saturate as the duration of the ionophoretic pulse exceeded 0.4sec. The tendency of the response duration and amplitude towards saturation might have been caused by a tendency in the rise of intracellular free calcium to approach saturation. Gorman and Thomas (1980) working on the R-15 neurone of Aplysia have showed that the rise in $[Ca^{2+}]_i$ achieved by ionophoretic Ca^{2+} pulses of constant amplitude reaches a plateau value at pulses lasting 50sec. In view of the fact that the volume of the R-15 neurone (radius of 150um) of Aplysia is about 50 times larger than of the mouse egg (radius of 40um) then it follows that in the mouse egg the rise in $[Ca^{2+}]_i$ will tend towards saturation at pulses of about 1sec in duration which are relatively close to the observed value of 0.4sec.

Simultaneous monitoring of potential and resistance revealed a decrease in membrane input resistance during the calcium-evoked hyperpolarization. The calculated reversal potential in the single experiment was -66mV.

Figure 19

The dependence of the duration and amplitude of the hyperpolarizing responses on the duration of the calcium current pulse in mouse eggs. The value of the prevailing membrane potential prior to a calcium injection is indicated by the number above the "resting" voltage baseline. The duration of the calcium current pulse is indicated by the number beneath the resting potential baseline.

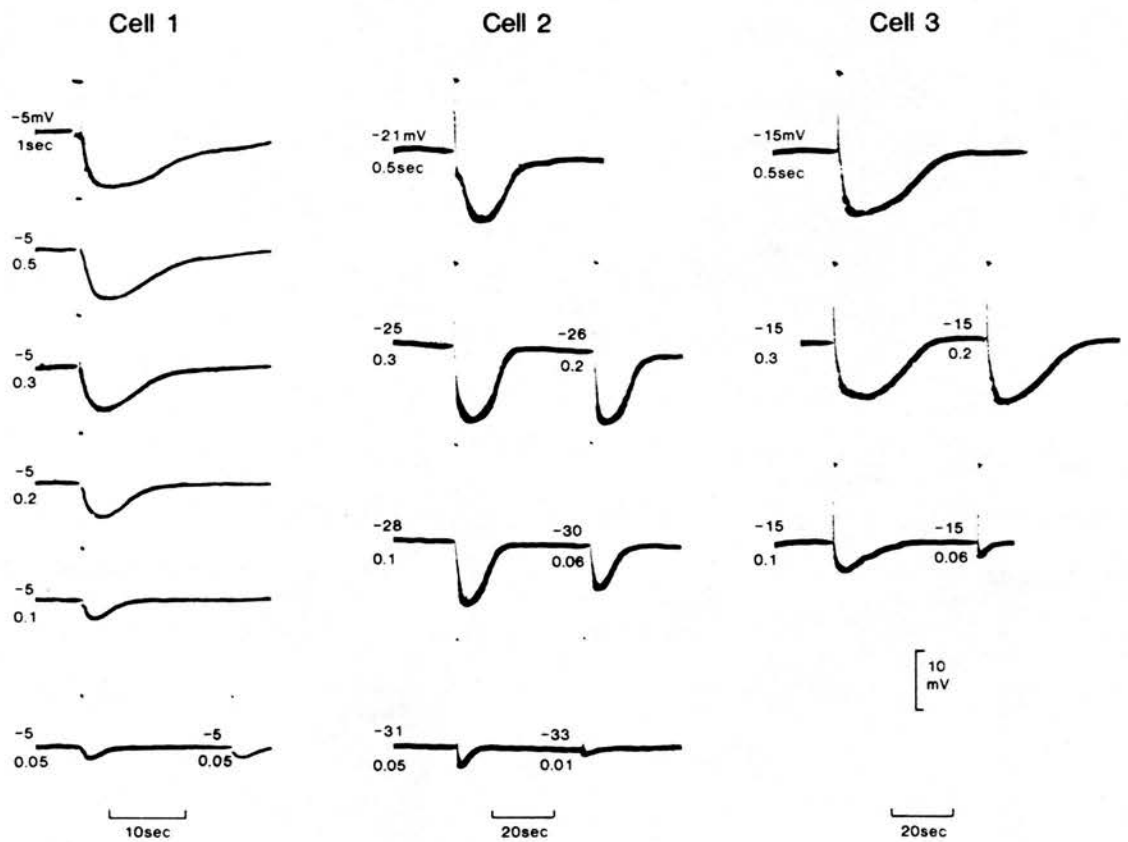
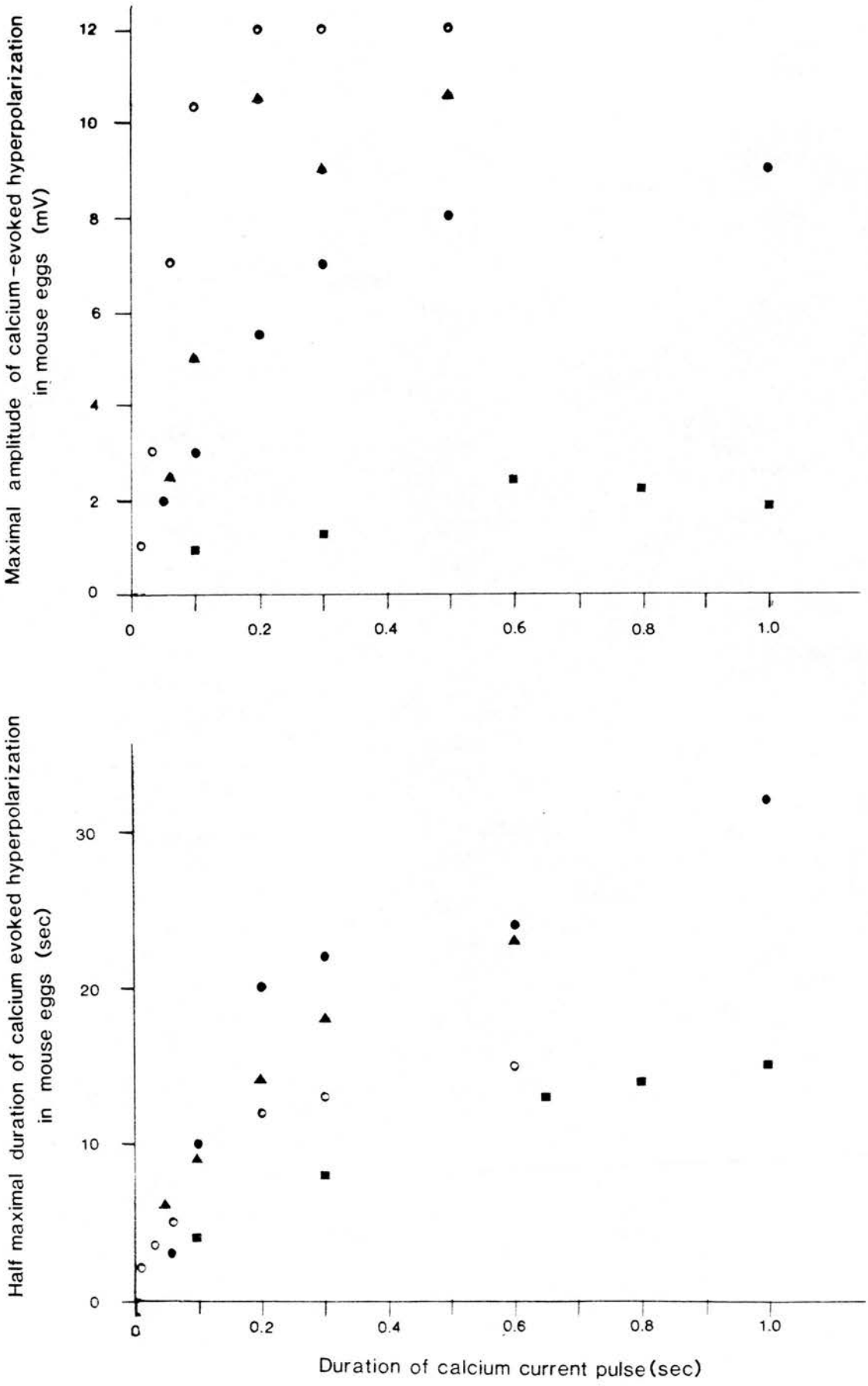

Cell 1
Cell 2
Cell 3
-5mV
1sec
-5
0.5
-5
0.3
-5
0.2
-5
0.1
-5
0.05
-5
0.05
10sec
-21mV
0.5sec
-25
0.3
-26
0.2
-28
0.1
-30
0.06
-31
0.05
-33
0.01
20sec
-15mV
0.5sec
-15
0.3
-15
0.2
-15
0.1
-15
0.06
10
mV
20sec

Figure 20

Plots of maximal (peak) amplitude and half-maximal duration of the responses (shown in Fig.19) against duration of the calcium current pulse. The filled circles refer to cell (1), the open circles to cell (2) and the filled triangles to cell (3). The filled squares refer to measurements taken from a cell not presented in Fig.19.


Maximal amplitude of calcium-evoked hyperpolarization in mouse eggs (mV)
12
10
8
6
4
2
0
0
0.2
0.4
0.6
0.8
1.0
Half maximal duration of calcium evoked hyperpolarization in mouse eggs (sec)
30
20
10
0
0
0.2
0.4
0.6
0.8
1.0
Duration of calcium current pulse (sec)

The ionic basis of the hyperpolarizing response was not attempted primarily because of the small size of the response and secondly because in any given cell both the amplitude and the duration of the calcium evoked hyperpolarization were found to vary widely for a series of constant calcium pulses.

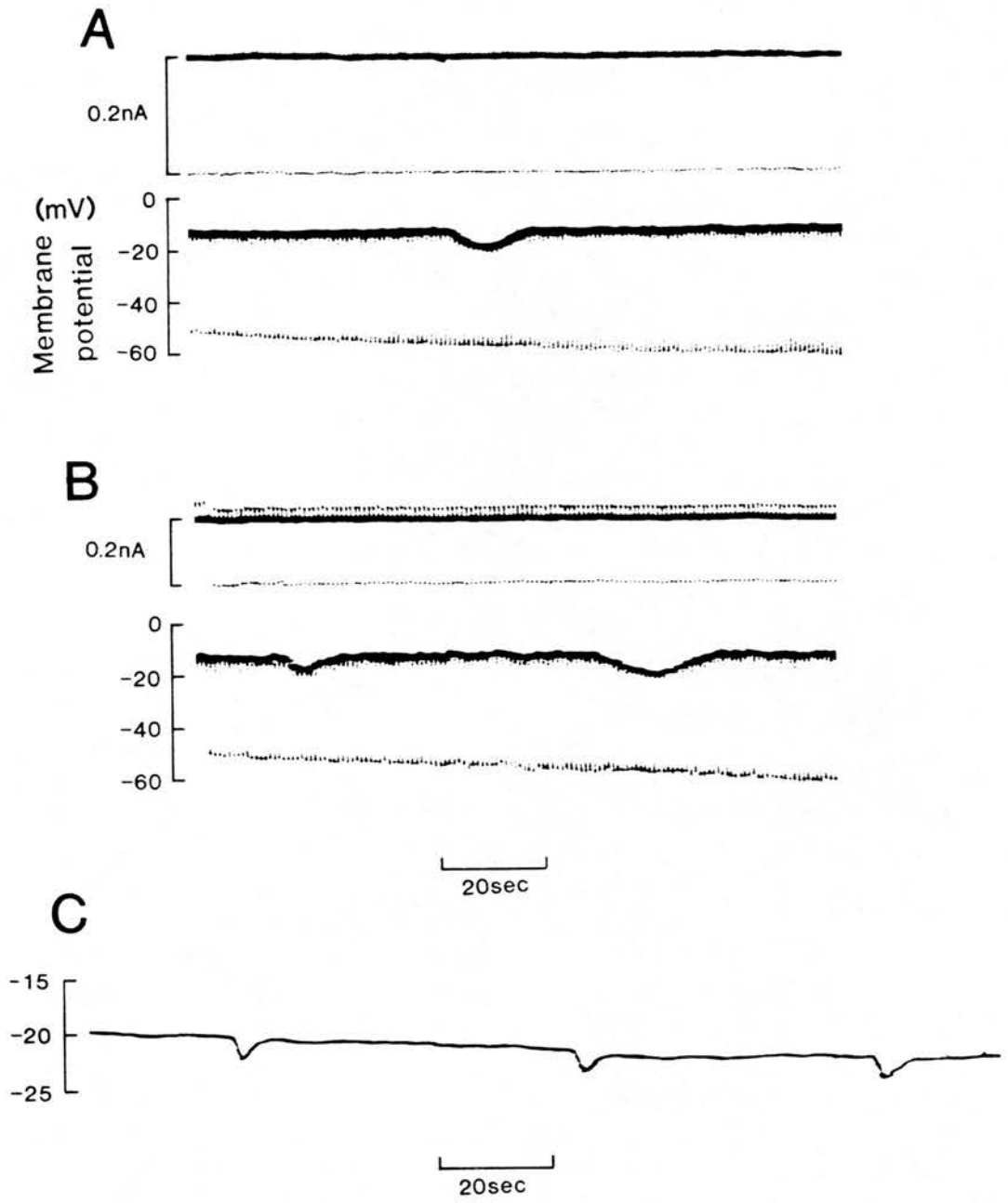
Section 10: Spontaneous hyperpolarizations in mouse eggs

In one experiment small recurring spontaneous hyperpolarizations were observed. This is shown in example (C) of Fig.21. In this particular example the spontaneous events were preceded by a calcium-evoked hyperpolarization.

However, in two other experiments (examples A & B of Fig.21) spontaneous hyperpolarizations were recorded by an intracellular microelectrode, without a calcium-injecting electrode being inserted into the egg, and in the presence of 2mM external lanthanum. The membrane input resistance fell during these hyperpolarizations as shown by the reduction in the size of electrotonic potentials, obtained in response to a constant current pulse. The calculated reversal potentials of these responses were -73(A) and -86(B)mV respectively. This strongly suggests an activation of a Ca^{2+}-activated K^{+} conductance but experiments are needed to exclude any possible role played by chloride ions.

Figure 21

Spontaneous hyperpolarizations observed in mouse eggs. A and B illustrate spontaneous hyperpolarizations recorded by K-acetate microelectrodes in different eggs in the presence of 2mM external lanthanum. C shows spontaneous hyperpolarizing responses in another egg that were recorded by a K-acetate electrode following a single ionophoretic pulse of calcium ions into another egg.


A
0.2nA
(mV)
0
-20
-40
-60
Membrane potential
B
0.2nA
0
-20
-40
-60
20sec
C
-15
-20
-25
20sec

DISCUSSION

Measurement of the true membrane potential in cells with high input resistance requires that the seal between the tip of the recording microelectrode and the plasma membrane is a tight one. To get a good estimate (to less than 5%) of what the real membrane voltage is, the ratio of the membrane conductance (g) to the leak conductance (g_L) must be very high (>20) as outlined below.

Assuming an equivalent circuit where the leak conductance (g_L) is in parallel with the membrane conductance (g) the observed membrane potential V is given by:

$$V = (E_L g_L + E g) / (g+g_L) \quad ...(4)$$

where E_L is the potential of the leak pathway

and E is the true membrane potential of the cell.

Rearrangement of equation 4 gives

$$V = [E_L(g_L/g)+ E]/[1+(g_L/g)]$$

Hence if $g_L << g$ then g_L/g tends to zero and V = E

In terms of resistance the true transmembrane potential will be recorded only when R/R_L tends to zero , where R is the true membrane input resistance and R_L is the resistance of the leak pathway (seal resistance).

Thus the presence of a leak conductance would seriously undermine membrane potential measurements in cells with high input resistances. If the insertion of a microelectrode into an egg which has a membrane potential of -75mV and an input resistance of 20MΩ,

induces a leak of 1nS (i.e. a giga-seal formation around the microelectrode tip) and assuming a value for the leak battery of -9mV, then according to equation 4, the observed value of the membrane potential would be -74mV. This represents only a 1.3% loss in the cell's membrane potential. However for a cell which has an input resistance of >400MΩ and a membrane potential of -75mV, the same values of g_L and E_L give a value of -56mV which represents a loss of 25% in the cell's membrane voltage.

The possible existence of a leak conductance pathway during the course of microelectrode recordings from hamster eggs was first pointed out by Miyazaki and Igusa (1982) who reported that high input resistances (400MΩ) were associated with the more negative membrane potentials (-50mv). In order for the membrane potential of a cell which has an input resistance of 400MΩ, to lie within 10% of its true value, the seal resistance should be at least 3,500MΩ. Membrane potentials of -75mV have been recorded in sea urchin eggs (Jaffe & Robinson ,1978 ; Chambers & de Armendi , 1979),which have diameters and input resistances (see Table 8) similar to those of hamster eggs. Indepedent estimates of the membrane potentials of the sea urchin egg derived from tracer flux measurements of the major extracellular ions (Jaffe & Robinson ,1978 ; Chambers and de Armendi,1979) are identical to those obtained by intracellular microelectrodes. The excellent agreement between the two methods provides convincing evidence

that at least in the sea urchin egg the seal resistance between the tip of the microelectrode and the egg plasma membrane must be of the order of several GΩ . An estimate of the seal resistance (R_L) can be obtained through the use of the following equation

$$R_L=R'(E_L-E)/(V-E) \quad .....(5)$$

which is derived from equation (4) by substituting $G-g_L$ for g (where $G=1/R'=g+g_L$), then solving for g_L and then inversing the result. In order to apply the above equation the following assumptions are made :- (a) the true membrane potential of the sea urchin egg E=-80mV which is equal to the equilibrium potential of $K^+(E_K)$ and (b) E_L=-10mV (see Weisenseel & Jaffe(1979) and Dascal, Landau & Lass (1984)). The values of R' and V used are those given in Table 8 i.e 600MΩ and -75mV respectively. Putting the above values into equation (5) the value of R_L is

$$R_L=0.6(-10+80)/(-75+80)=8.4G\Omega$$

Thus the seal resistance during intracellular recordings from sea urchin eggs is high suggesting that the recorded membrane potential is close to its true value.

If the true value of the membrane potential for hamster eggs is also equal to the equilibrium potential for K^+ of -80mV (see Chapter 3)and given that E_L=-10mV (see section 2 in Part I of this chapter), V=-34mV (actual mean recorded membrane potential;see Table 6 in section I of this chapter) and R'=290MΩ (mean value 287±166MΩ;see Table 6 in section 2 of this chapter) then

R_L=290(-9+80)/(-34+80)=440MΩ≙0.4GΩ. Such a low seal resistance suggests that the recorded mean membrane potential is not the true membrane potential. However the highest recorded membrane potential was -51mV(corrected value); the corresponding value of the input resistance at -51mV was 620MΩ. Putting these values for V and R' in equation (5) a value of R_L=1.5GΩ is obtained. This is less than 8.4GΩ , the estimated seal resistance of the recordings from sea urchin eggs. Given that such a high seal resistance could be achieved in hamster eggs the recorded membrane potential according to equation (5) could be -72mV substiantially higher than the observed value of -51mV.

The specific membrane resistance of hamster eggs compares favourably with the specific membrane resistance of other eggs (see Table 8). The presence of relatively non-leaky membranes in eggs, serves probably to minimize the possibility of parthenogenetic activation by agents such as calcium. The latter is known to cause resumption of the developmental process (i.e. activation) in sea urchin eggs (Steinhardt & Epel ,1974), after exposure to the calcium ionophore A23187. Parthenogenetic activation can also be induced in mouse eggs by intracellular calcium injections (Fulton & Whittingham , 1978).

The calcium injection experiments have shown that a rise in the intracellular concentration of free calcium ions in hamster eggs, causes a hyperpolarizing response with a concomitant rise in the membrane input

Table 8. The electrical properties of unfertilized eggs of different species.

Preparation	Membrane potential (mV)	Input resistance (MΩ)	Egg diameter (μm)	Apparent surface area (cm^2)	Specific membrane resistance ($K\Omega cm^2$)	Specific membrane capacitance ($\mu F/cm^2$)	Experimental temperature ^oC	Reference
Sea urchin	-75±3	640±90	80	$2x10^{-4}$	128±18	1.1±0.2	15	Jaffe & Robinson (1978) Jaffe, Hagiwara & Kado (1978)
Sea urchin	-76±0.8	540±60	80	$2x10^{-4}$	108±12	-	22 25	Chambers & de Armendi (1979)
Xenopus	41.1±5.8	0.714±0.232	140±70	$4.1x10^{-2}$	28.6±6.2	6.3±1.1	18 24	Kusano, Miledi & Stinnakre (1982)
Immature Bufo	-70	-	-	-	25	6-11	not specified	Maeno (1959)
Annelid	-60	100	80	$2x10^{-4}$	20	-	20-22	Hagiwara & Miyazaki (1977)
Tunicate	-3.0±4.9	56.4±20.3	250-300 mean =275	$2.4x10^{-3}$	135±49	0.95	9-14	Miyazaki, Takahashi & Tsuda (1974)
Mouse	-35.7±2.6 (at a $[Ca^{2+}]_o$ of 20mM)	50 200	72±3	$1.6x10^{-4}$	8.0 32.5	1.5±0.2	30-33	Okamoto, Takahashi & Yamashita (1977)

Hamster	-29±7	142±65	72	1.6×10^{-4}	22.7±10.4	3.4±0.6	30-32	Miyazaki & Igusa (1981; 1982).

Apparent surface area refers to the area of a smooth sphere.
Specific membrane resistance and capacitance were calculated using the value of the apparent surface area.
Data are expressed as mean±SD where possible.

conductance. This conductance change in hamster eggs seems to be primarily selective for potassium ions. Such a calcium mediated opening of potassium selective membrane channels has been proposed to be responsible for the sperm evoked periodic hyperpolarizations observed to occur following sperm-egg fusion (Miyazaki & Igusa, 1982 ; see Table 1 in section 3 of the Introduction)

Igusa and Miyazaki (1983) have observed that the timecourse of the calcium-evoked hyperpolarization was greatly prolonged by a drop of 5°C (viz. from 31 to 26°C) in the temperature of the solution bathing the eggs. They attributed the prolongation of the response to a reduction in the calcium buffering power of the egg at the lower temperature. The same explanation can be advanced to account for the apparent discrepancy between the mean response duration of 11.2±1.8sec(n=65) obtained in an earlier study by Miyazaki and Igusa (1982) , who conducted their experiments at 33°C and the value of 35±27sec(n=61) obtained from the experiments of this study which were carried out at 23°C. Alternatively the observed difference in the timecourse of the response might reflect a difference in the inactivation kinetics of the channels since the rates at which channels open and close are known to be affected by changes in temperature (Hodgkin & Huxley , 1952). The relatively lower experimental temperature employed in this study is probably also responsible for the considerably higher values of input

resistance recorded as compared to the value of 140MΩ reported by Miyazaki and Igusa (1982). Membranes, at certain critical temperatures (melting point temperature), are known to undergo lipid-phase transitions from a relatively fluid state to a more compact liquid- crystaline state (Chapman,1975). It is therefore feasible that the higher input resistance values observed at the relatively lower temperature of 23^{o}C are caused by structural changes of the egg membrane. Since most of the ionic current flow imposed by either a hyperpolarizing or a depolarizing current pulse must cross the cell membrane via ionic channels, a rise in the resistance of the membrane to ion flow must mean either a decrease in the conductance of individual channels and/or a reduction in their open state probability. In fact a reduction of the open state probability of Ca^{2+}-activated K^{+}-channels caused by a fall in temperature has recently been reported by Barret,Magleby and Pallotta (1983) in rat skeletal muscle cells.

The hyperpolarizations produced by the insertion of the calcium injection pipette and, on certain occasions, by insertion of pipettes containing other solutions (eg. cobalt chloride) is probably due to a transient calcium influx through the leak conductance pathway that must be generated across the plasma membrane at the instant of impalement. This interpretation is in agreement with the results of Lassen, Pape, Vestergaard-Bogind and Bengston, (1974) who found that the process of electrode

insertion into Amphiuma red cells produces a similar transient hyperpolarization that requires the presence of external calcium. Leakage of calcium ions from the tip of the micropipette filled with $CaCl_2$ might also be a contributing factor in producing these "impalement" responses in some experiments.

The number of K^+-channels that open in response to a calcium injection can be estimated from the observed change in conductance (g_p-g) during a response, provided a value for the single channel conductance (γ_K) is known. In most other preparations γ_K has been found to range from 100 to about 200pS under symmetrical K^+ solutions across the cell membrane (see reviews by Lattore & Miller, 1983; Petersen & Maruyama, 1984). Since the calcium injections were performed into eggs which were under physiological K^+ gradients, i.e. asymmetrical, the lower values of 100pS is probably more representative of γ_K during a response. The number of open channels N is equal to the ratio of g_K/γ_K where $g_K = g_p - g$. A plot of the calculated values of g_K for the individual data in Table 7 (see section 4 in Part II of this chapter) gave a median value of 18nS. However , the plot also showed a small secondary peak around 85nS. As 70% of the values were distributed around 18nS. this value should approximate to g_K. Possibly a more reliable approach to the estimate of g_K would be to discard the results where responses had large time constants ,say >10sec for the recovery of the input conductance (see

Chapter III). On this basis the estimated value of g_K is 21nS. If g_K=20nS then the number of open channels (N) during a calcium-evoked response is about 200. If the single channel is similar to that found in neurones Helix pomatia (Lux, Neher & Marty, 1981), i.e. 19pS, then the number of open channels during a response becomes approximately 1050. Igusa and Miyazaki (1983) have reported that calcium injections into a hamster egg, via two different microelectrodes situated 50μm apart, interfered with each other in that the first calcium injection delivered via one of the calcium microelectrodes produced a full response whereas the second calcium injection delivered via the second microelectrode resulted in a diminished response. Hence Igusa and Miyazaki inferred that the increase in $[Ca^{2+}]_i$, responsible for generating the full response, involved nearly the whole inner surface of the egg membrane. If this is true then the channel density must be approximately either 1 channel per 100um^2 if γ_K=100pS or 1 channel per 20um^2 if γ_K=19pS. Neither estimate allows for the extra surface area due to the microvilli. If channels are also present in the microvilli then the channel density falls to about 1 channel for every 300um^2 (if γ_K=100pS) or to 1 channel for every 63um^2 (if γ_K=19pS). In contrast Walsh and Singer (1983) have estimated, for vertebrate smooth muscle a channel density for the Ca^{2+}-activated K^+-channels of 1 channel per 5 um^2 (i.e. 2000 channels per cell; surface area of a smooth muscle cell $\simeq 10^4 \mu m^2$).

Flanagan and Pearson (1983) have estimated that the number of these channels per pancreatic acinar cell to lie between 25-60 (mean±SD 45±11). Since a single acinar cell has an area of 600μm^2 (Bolander 1974) this corresponds to a channel density of 1 channel per 13μm^2.

A possible explanation for the much smaller hyperpolarizations observed in mouse eggs (average of 6mV as compared to 27mV for hamster) in response to calcium injections could be that there are perhaps fewer channels present in mouse eggs. Thus for any given activating calcium concentration one would expect to see a larger hyperpolarization in hamster eggs than in mouse eggs, assuming that the open state probability of the channels is approximately the same in both cases. Alternatively the sensitivity of the channels to changes in intracellular free calcium might differ considerably from hamster to mouse.

The magnitude of the outward potassium current that must flow through the channels when they are activated is given by:

$I_K = (E_K - V)g_K$ where

I_K is the potassium current

E_K is the potassium equilibrium potential

V is the membrane potential

g_K is the membrane potassium conductance

Given that the more reliable estimates for the potassium equilibrium potential and of g_K are -80mV and 20nS respectively (see Discussion of Chapter III)

and given a resting potential of -25mV (see Table 7, section 4 in Part II of this chapter) the expected outward potassium current is approximately equal to 1nA. A current of 1nA corresponds to an efflux of approximately 1.0×10^{-14} moles of potassium ions per second. At 5mM external potassium concentration and an equilibrium potential of -80mV the effective intracellular potassium concentration must be about 120mM. Hence an efflux of 1.0×10^{-14} moles/sec would change the intracellular potassium concentration by about only 1% if it persisted for 30sec (average response duration). That the effective $[K]_i$ is not appreciably altered during a calcium-evoked response is further supported by the observation that no change in the reversal potential of the response occurs (excluding responses where substantial membrane breakdown is evident) after repeated calcium injections.

The value for the effective $[K^+]_i$ of about 120mM, (given that E_K=-80mV) is in good agreement with the value obtained by Miyazaki and Igusa(1982). These workers estimated the effective $[K^+]_i$ to be about 140mM (given an estimated value for the reversal potential of about -85mV at $[K^+]_o$=5mM).

On the other hand Powers and Tupper (1975) have estimated the effective $[K^+]_i$ in <u>mouse</u> eggs to be about 200mM. An apparent discrepancy therefore exists between the estimate of $[K^+]_i$ derived by Powers and Tupper for mouse eggs and the estimates derived for

hamster eggs from measurements of the reversal potential. It may be that this is a true species difference but more experiments are needed to resolve the issue.

The generation of hyperpolarizing responses by the application of large hyperpolarizing current pulses in the presence of external lanthanum probably indicates that they are caused by calcium influx through "membrane pores" that are created during and following the period of electrical stimulation. A similar mechanism can also be advanced to explain the generation of hyperpolarizing responses in macrophages (Gallin, Wiederhold, Lipsky & Rosenthal, 1975) and fibroblasts (Nelson, Peacock & Minna, 1972 ; see also Table 1 in Introduction) upon stimulation of these cells with large current pulses.

CHAPTER III

THE EFFECT OF MULTIVALENT IONS AND DINITROPHENOL (DNP) ON THE CALCIUM-EVOKED HYPERPOLARIZATION

INTRODUCTION

Injections of calcium into the hamster egg causes a transient hyperpolarization of the egg's plasma membrane. It has been shown (Miyazaki & Igusa,1982; Igusa & Miyazaki 1983; Georgiou, Bountra, Bland and House, 1983) that the hyperpolarizations are mainly caused due to the opening of potassium channels which are activated by an increase in the activity of intracellular calcium. Little is known, however, about the recovery phase of these calcium-induced hyperpolarizations. This chapter is primarily concerned with the way certain multivalent ions modify the recovery phase of the calcium- evoked response.

METHODS

The methods of obtaining and treating eggs, of intracellular recording and of injecting ions into eggs were the same as already described in the Methods section of chapter two. The bathing solutions used in these experiments are given in Table 4 of Chapter II.

RESULTS

Section 1: Intracellular injection of multivalent ions

In Chapter II evidence was presented which seemed to indicate the presence of a serious leak artifact during microelectrode impalements in hamster eggs. The

observed membrane potential in the presence of such a leak pathway is given by

$$V = (E g + E_L g_L)/ (g+g_L) \ldots . (1)$$

where E_L = leak pathway potential

E = membrane potential

g_L = leak conductance

g = membrane conductance

and the observed membrane input resistance by

$$R' = R\ R_L / R+R_L$$

or in terms of conductances by

$$g' = g_L + g$$

Hence the observed input conductances (g') is a lump parameter, its value depending on the magnitude of the leak conductance introduced by the microelectrode.

Hamster eggs possess potassium channels which open in response to a rise in the concentration of cytosolic calcium (see Chapter II, Part II). However care must be taken in demonstrating the activation of these channels since in addition to the leak pathway introduced at the site of electrode penetration for calcium injection, it was found that application of a large ionophoretic pulse of ions (i.e. 2 nA, deflecting the membrane potential to between + 150 to + 200 mV) also caused temporary membrane breakdown thus adding yet another shunt pathway for current flow. This additional leak conductance, g_{il}, could lead to a serious underestimation of the reversal potential of the response of the hamster egg to calcium injection if

both processes, namely activation of channels and formation of g_{il} coincide in time. Furthermore if the time course of membrane repair exceeds the time course of potassium channel inactivation it could lead to distortion of the response recovery profile.

Evidence suggesting the presence of an injection leak conductance pathway comes from low reversal potentials (-30 to -50mV) in response to calcium in some 20% of the experiments . The reversal potential was calculated according to equation (3) of Chapter II. These responses consisted of a relatively brief hyperpolarization (10-20sec) which was followed by a pronounced depolarization lasting several minutes. Both the hyperpolarizing phase and much of the subsequent depolarizing phase were associated with a considerable fall of the membrane input resistance. A semilogarithmic plot (see example shown in Fig.22a) of the of the recovery of the % loss in input resistance (i.e. R_o-R_t/R_o (Meech, 1974) where R_o is the input resistance at rest and R_t is the input resistance at time t after the injection of calcium) produced a time constant, T_R , which was almost always of the order of minutes (see Table 9).

In contrast to these results calcium-induced responses with short recovery time constants for the input resistance (T_R < 10 sec) were almost invariably associated with high reversal potentials (E_r>-70mV). Representative examples illustrating the striking differences between these two kinds of responses are

Figure 22a

Semilogarithmic plot of (R_o-R_t/R_o) against time. The reversal potential for this example was -52mV and the time constant (T_R) for the recovery of the input conductance, calculated from the linear region of the graph was 120sec.

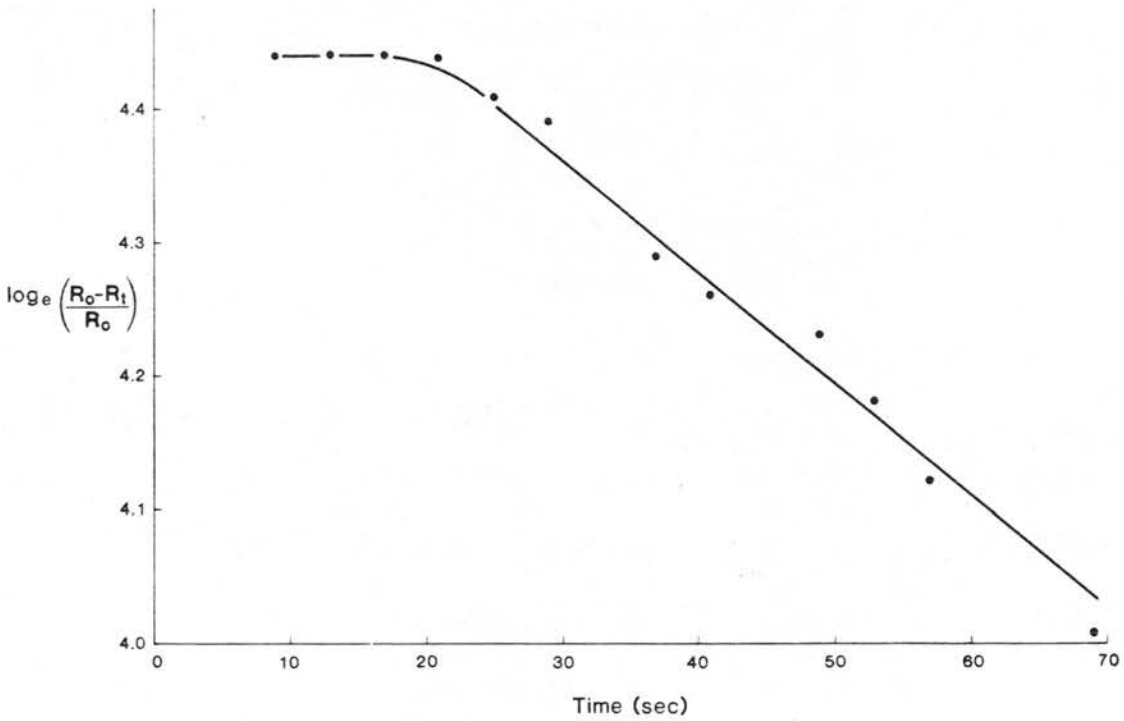

4.4
4.3
4.2
4.1
4.0
$\log_e \left(\frac{R_o - R_t}{R_o}\right)$
0
10
20
30
40
50
60
70
Time (sec)

Table 9

Relation between the reversal potential (E_r) of the calcium-evoked response and the time constant (T_R) for the recovery of the % loss in input resistance $\left(\frac{R_o - R_t}{R_o}\right)$ in 43 individual responses obtained from 23 eggs.

Cell No.	Reversal potential (E_r) (mV)	Time constant (T_R) (sec)
1	-62	22.0
	-75	5.5
	-78	5.0
2	-55	65.0
	-34	350.0*
3	-58	84.0
	-70	14.0
4	-81	6.0
5	-77	9.0
	-81	5.0
6	-73	6.5
7	-42	180.0*
8	-61	11.0
	-79	11.0
9	-58	25.0
10	-67	16.0
	-68	6.0
11	-64	27.0
12	-86	8.0
	-45	67.0
13	-87	7.0
14	-52	120.0

Cell No.	(mV)	(sec)
15	-61	38.5
	-67	17.0
16	-53	138.0
	-70	21.0
17	-39	380.0*
	-76	10.0
18	-70	15.0
	-44	19.0
19	-69	45.0
20	-38	38.5
21	-58	14.5
22	-71	13.5
23	-78	31.5
24	-57	42.0
	-68	20.0
	-43	120.0
25	-67	24.0
	-73	11.0
	-65	9.0
26	-69	22.5
	-30	187.0*

Results indicated by the asterisk are not presented in the graph of Figure 23.

shown in Figure 22b. It seems plausible therefore that responses with low reversal potentials indicate a form of non-selective membrane breakdown caused by the ionophoretic calcium pulse. During a calcium injection the membrane potential is usually displaced to +150mV which is in the strongly outward rectifying region (Miyazaki & Igusa, 1982) of the current voltage relation. This might cause an indiscriminate ion flow across the membrane via perhaps a "punch-through" mechanism (Coster, 1965).

If the responses with low reversal potentials were the result of the creation of a variable time-dependent leak conductance pathway one would expect to find a correlation between the value of the reversal potential (E_r) and the time constant for the recovery of the input resistance (T_R). A regression analysis of the results presented in Table 9 which are also shown graphically in Figure 23, produced a good correlation between E_r and T_R ($r = 0.65$ for 41 d.o.f).

The second piece of evidence that lends further support to the injection leak hypothesis comes from ionophoretic pulses of multivalent ions other than calcium or strontium. Electrophoretic application of either $K^+(n=2)$, $Mg^{2+}(n=2)$, $Co^{2+}(n=1)$, $Ba^{2+}(n=2)$ or $La^{3+}(n=1)$ failed to produce any hyperpolarization but instead caused slight depolarizations with a concomitant fall in the cell's input resistance. Typical examples of such responses are shown in Figure 24. The reversal potentials of the

Figure 22b

Variation of the profile of hyperpolarizing responses obtained in response to calcium injections. A and B were obtained from the profile of hyperpolarizing responses obtained in response to calcium injections. A and B were obtained from the same cell and in response to identical calcium current pulses.

Notice that in B the value of the potential at the peak of the response is lower than that of A and is accompanied by a much larger drop in input resistance, both signs of contamination by an injection-induced leak conductance (g_{il}). C illustrates a response obtained in another egg and thought to be contaminated by g_{il}. The reversal potentials of the responses shown in A, B and C were -39 , -30 and -45mV respectively. By contrast the responses displayed in D (obtained from the same egg) are believed to not have suffered substantially by the formation of g_{il} during the ionophoretic calcium pulse. Notice that the peak value of the membrane potential in the two responses shown in D is considerably higher than in the responses shown in A, B or C. The recovery of the input resistance from its low value during the response back to its original value is also appreciably faster in D as compared to A, B or C. The reversal potential of the responses illustrated in D were -70mV for the calcium evoked hyperpolarization (closer on the left) and -73mV for the one on the right.

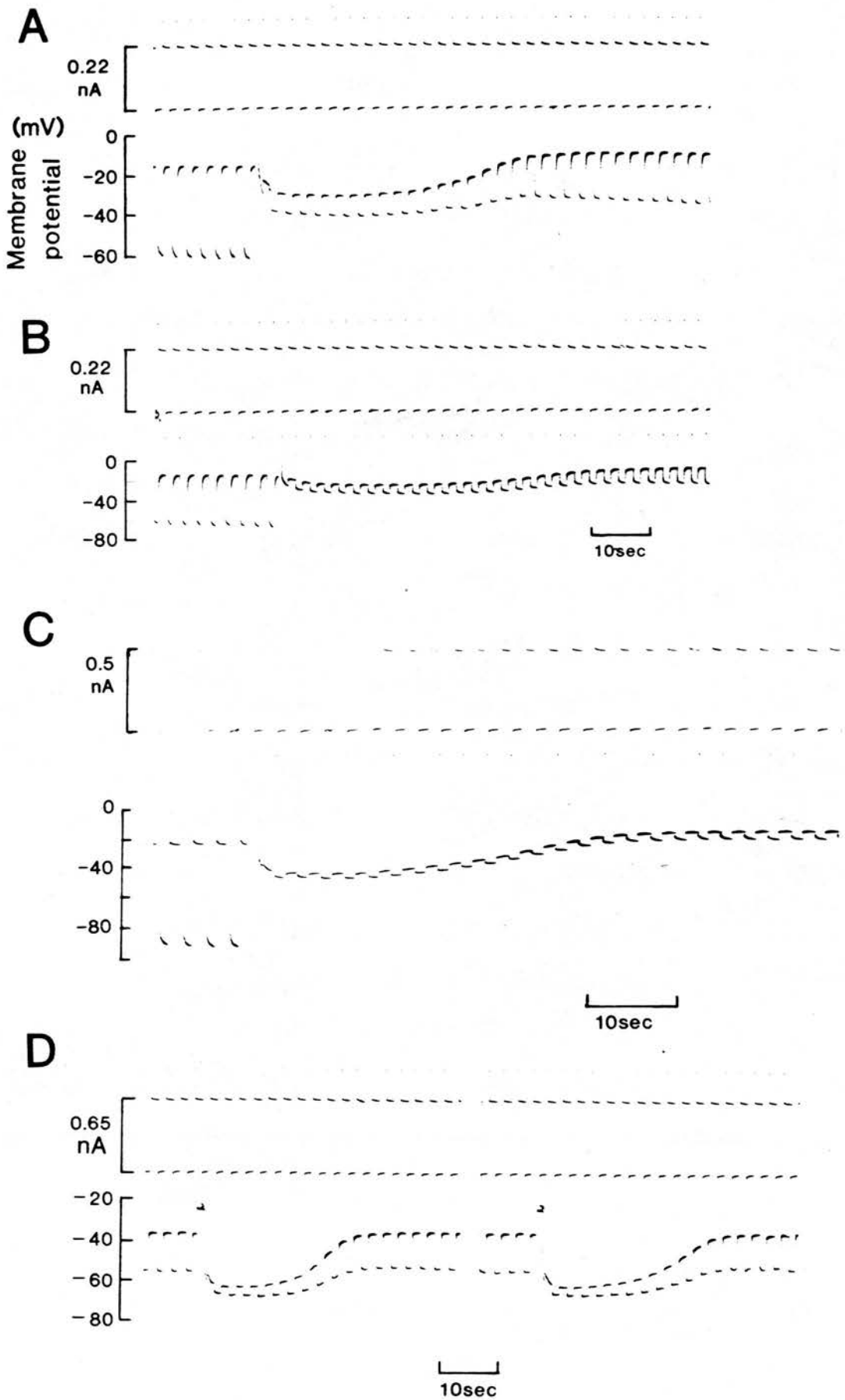

A
0.22
nA
(mV)
Membrane potential
0
-20
-40
-60
B
0.22
nA
0
-40
-80
10sec
C
0.5
nA
0
-40
-80
10sec
D
0.65
nA
-20
-40
-60
-80
10sec

Figure 23

The relationship between the time constant(T_R) of the % loss in input resistance (i.e. increase in conductance) and the reversal potential(E_r) of the calcium evoked hyperpolarization.

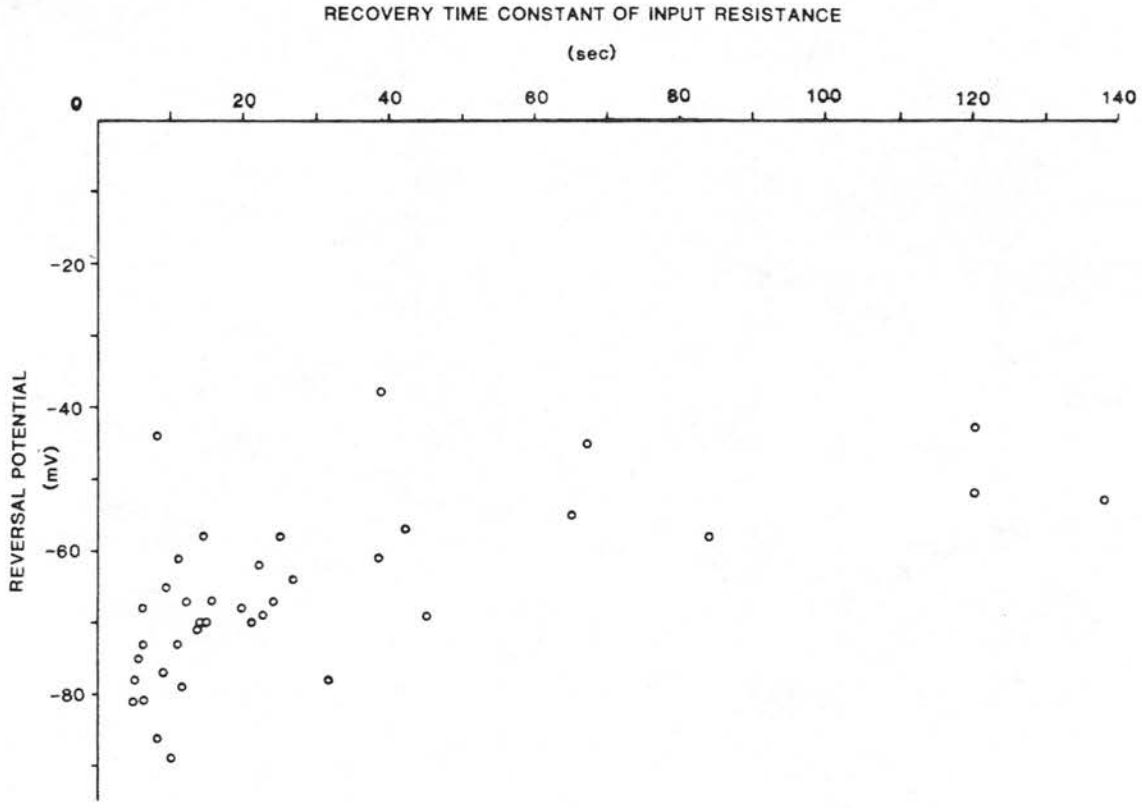

RECOVERY TIME CONSTANT OF INPUT RESISTANCE
(sec)
0
20
40
60
80
100
120
140
-20
-40
-60
-80
REVERSAL POTENTIAL
(mV)

Figure 24

The effect of multivalent cation injections on the membrane potential and resistance of hamster eggs.

The effects produced by injecting Ba^{2+} are illustrated in A. B shows the membrane response to an injection of Co^{2+} and C shows the membrane reaction in response to an injection of La^{3+}. Notice that in all three examples the membrane potential underwent a slight depolarization following the ionophoretic pulse , associated with a reduction in input resistance as judged by the reduction in the size of elecrotonic potentials generated in response to constant current pulses (top traces of A, B and C)

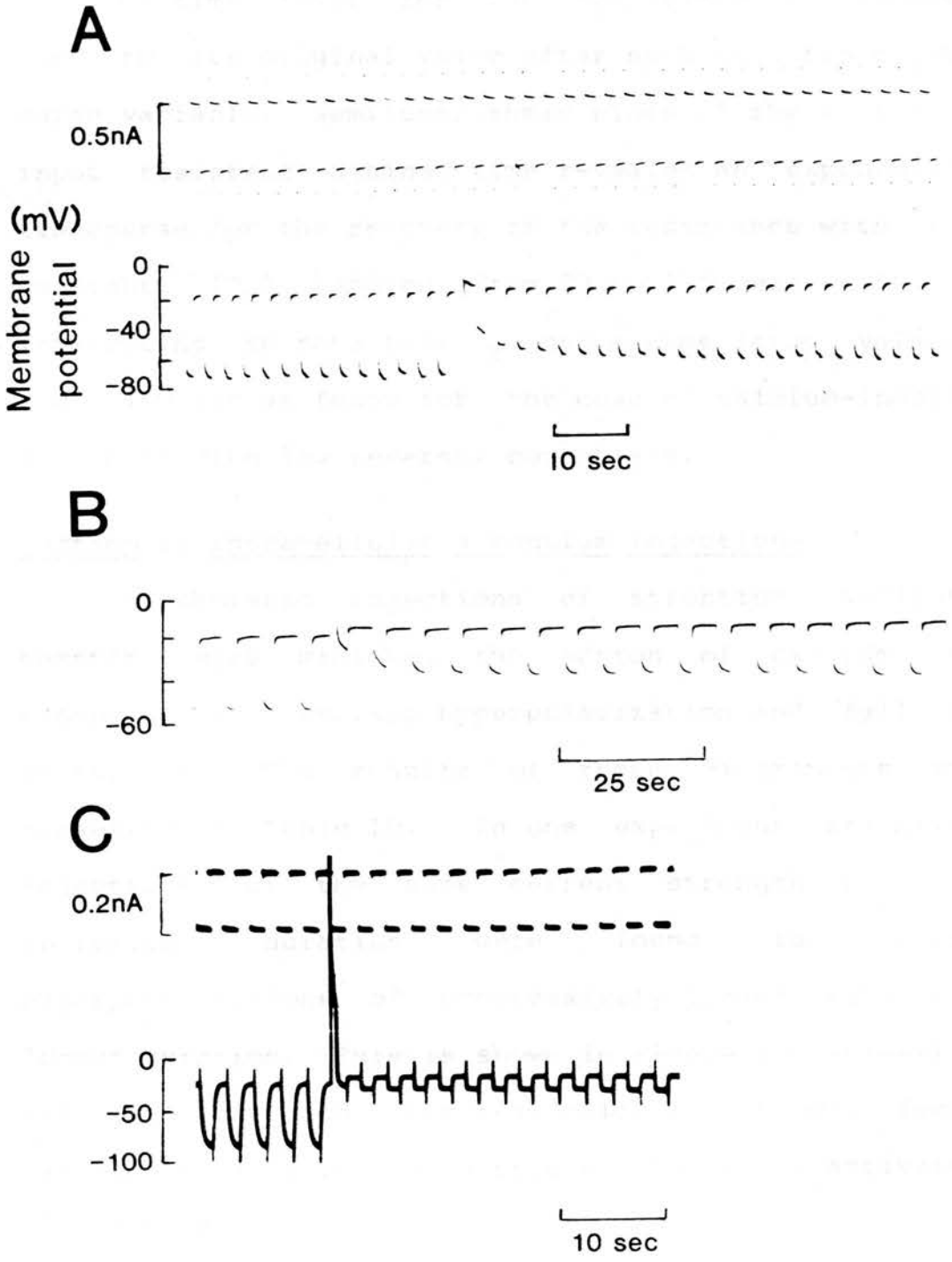

A
0.5nA
(mV)
Membrane potential
0
-40
-80
10 sec
B
0
-60
25 sec
C
0.2nA
0
-50
-100
10 sec

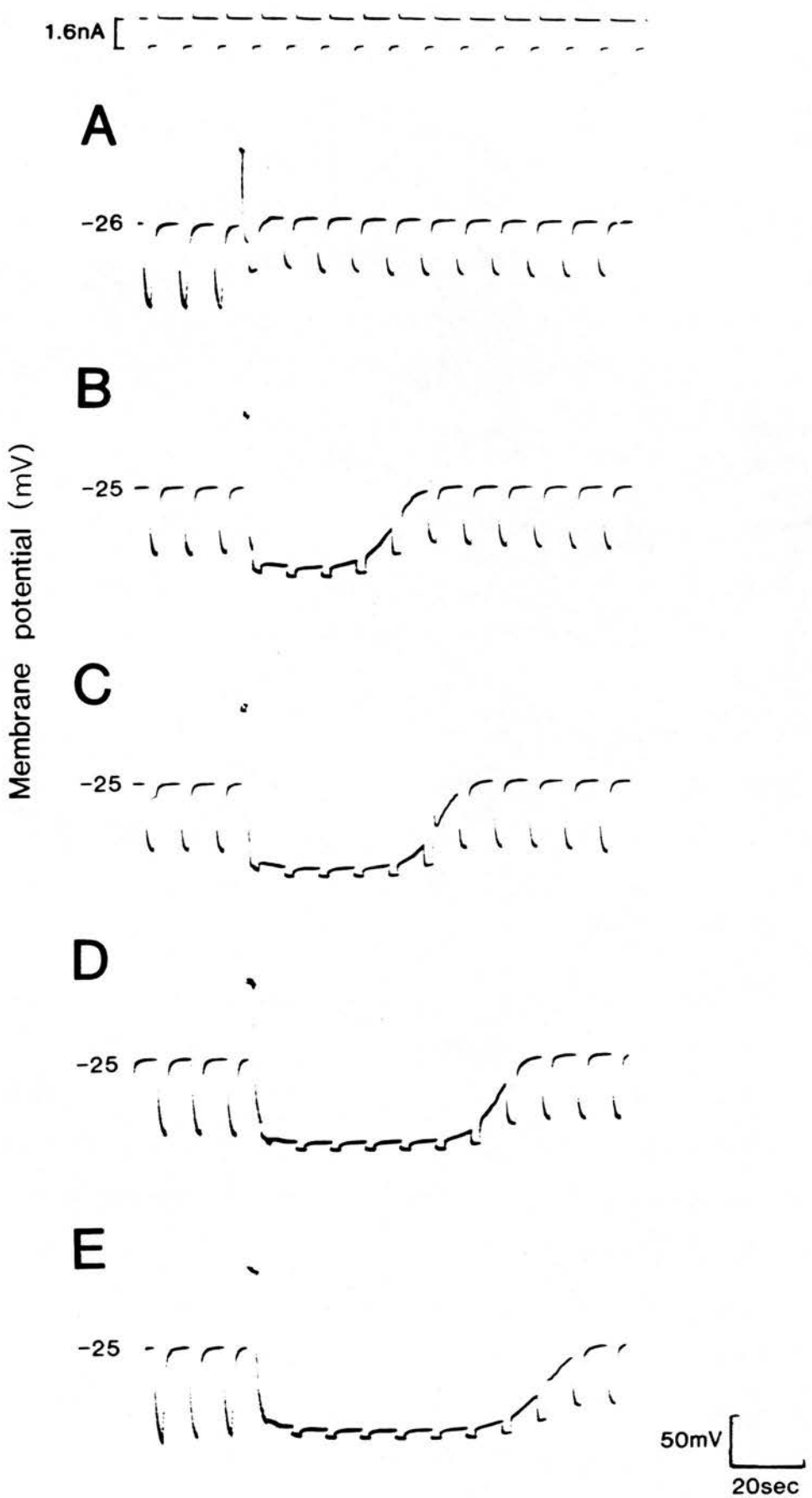
Table 10

The effect of intracellular Sr^{2+} injection on the electrical properties of unfertilized hamster eggs.

Cell No.	Recorded membrane potential (V) (mV)	Amplitude (ΔV) of Sr^{2+}-induced hyperpolarization (mV)	Reversal potential (E_r) of the Sr^{2+}-induced hyperpolarization (mV)	Input resistance prior to Sr^{2+} injection (MΩ)	Input resistance at peak of the hyperpolarization (MΩ)
1	-25	22	-87	255	28
2	-16	8	-70	138	65
3	-30	15	-77	133	62
4	-35	16	-58	285	89
5	-24	24	-75	148	93
6	-17	38	-88	100	47
Mean±SD	-24.5±7.0	20.5±10.3	-76±11	176±75	62±23

Figure 25

The effect of the duration of a constant strontium current pulse on the time course and size of the resulting hyperpolarization. The duration of the strontium current pulse was 0.1,0.2,0.4,0.6 and 1.0 sec for A, B, C, D and E respectively. The voltage deflections in the traces A to E are electrotonic potentials evoked in response constant current pulses (uppermost trace) for continous monitoring of the input resistance.


1.6nA
A
-26
B
-25
C
-25
D
-25
E
-25
Membrane potential (mV)
50mV
20sec

Section 3: The effect of different multivalent ions on the resting potential and resistance

Application of external lanthanum (n=14)always brought about a steady hyperpolarizing shift in the cell's membrane potential. This shift was invariably accompanied by at least a doubling in the cell input resistance. Representative records of the hyperpolarizing action exerted by lanthanum are shown in Figure 26.

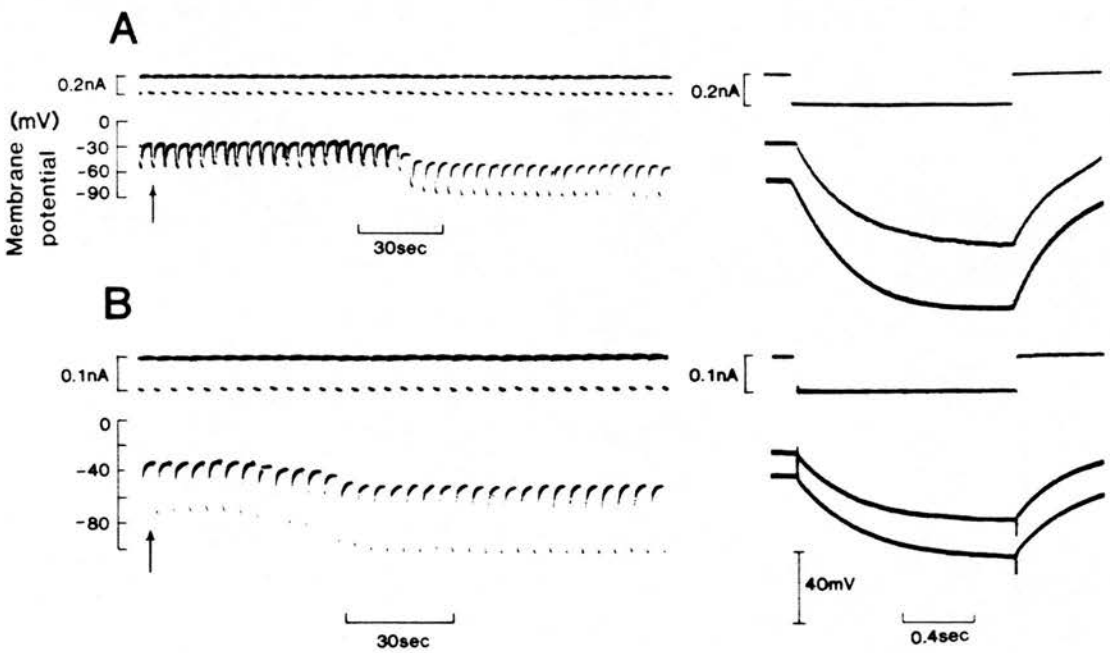
This "stabilising" action of lanthanum was fully reversible, the membrane potential and resistance assuming their original values upon replacement of external lanthanum with normal lanthanum-free solution.

The mean(±SD) value of the membrane potential and the input resistance in lanthanum solutions was -44±13mV (n=14 cells, range between -21 to -65) and 432±191MΩ (range between 160-760MΩ) respectively. In accordance with the findings reported in Chapter II high membrane potentials were associated with high input resistances.

In one experiment an alteration of the bathing medium from normal solution to one containing 20mM calcium caused a steady hyperpolarization (from -23 to -30mV) which was associated wirh an increase in input resistance (from 87 to 113MΩ). In 12 experiments where membrane potential and resistance measurements were performed on cells bathed in a 10mM calcium solution, the average(±SD) values obtained were -27±9mV and 243±117MΩ . These values are not signifantly different

Figure 26

The increase in membrane potential and input resistance produced by bathing hamster eggs in solutions containing lanthanum. In both A and B the left hand trace is the pen-trace of the experiment, the arrows indicating the time at which perfusion of the experimental chamber with lanthanum containing solutions began. The traces on the right are oscilloscope pictures taken at a much faster time base showing the changes in membrane potential which occured in response to a single identical current pulse (shown on top of the voltage trace of each record) in the presence of external lantanum.


A
0.2nA
(mV)
0
-30
-60
-90
Membrane potential
30sec
0.2nA
B
0.1nA
0
-40
-80
30sec
0.1nA
40mV
0.4sec

from that obtained in 4mM calcium (see page 35,36). High magnesium solutions (n=5) also had no detectable effect on potential and resistance.

Section 4: The enhancing effect of lanthanum on the calcium-evoked hyperpolarization

The effects of either external lanthanum or high calcium were investigated with the following procedure. First two or three calcium-evoked hyperpolarizations of similar duration and amplitude were obtained in normal solution. This was done so as to minimise the possibility that any subsequent change in either the time course or amplitude of the response was caused by variation in the amount of calcium ejected from the pipette rather than by the action of lanthanum or calcium on the cell membrane. Then calcium injections were repeated in lanthanum containing solutions and finally in normal solutions to ensure recovery from lanthanum treatment. In two experiments this procedure could be repeated in the same cell several times. A representative example of one such experiment is shown in Figure 27.

In all four experiments carried out, the results of which are presented in Tables 11A,11B and 11C externally applied lanthanum greatly prolonged the calcium-induced hyperpolarization (see measurement of time to half maximum amplitude, $T_{1/2}(V)$ in Table 11B). Restoration of the original timecourse of the response occurred after removal of external lanthanum. A characteristic feature of responses obtained in lanthanum solutions

Figure 27

The effect of external lanthanum on the profile of the calcium-evoked hyperpolarization. All the records presented in this figure were obtained from the same egg. A shows calcium-evoked responses obtained in normal solution whereas B shows responses obtained in a solution containing 1mM lanthanum. Recovery from lanthanum treatment is presented in C. D shows that readmission of lanthanum caused a return of the response profile to one similar to those illustrated in B. The voltage deflections are electrotonic responses produced in response to constant current pulses (not shown) for continous monitoring of the input resistance.

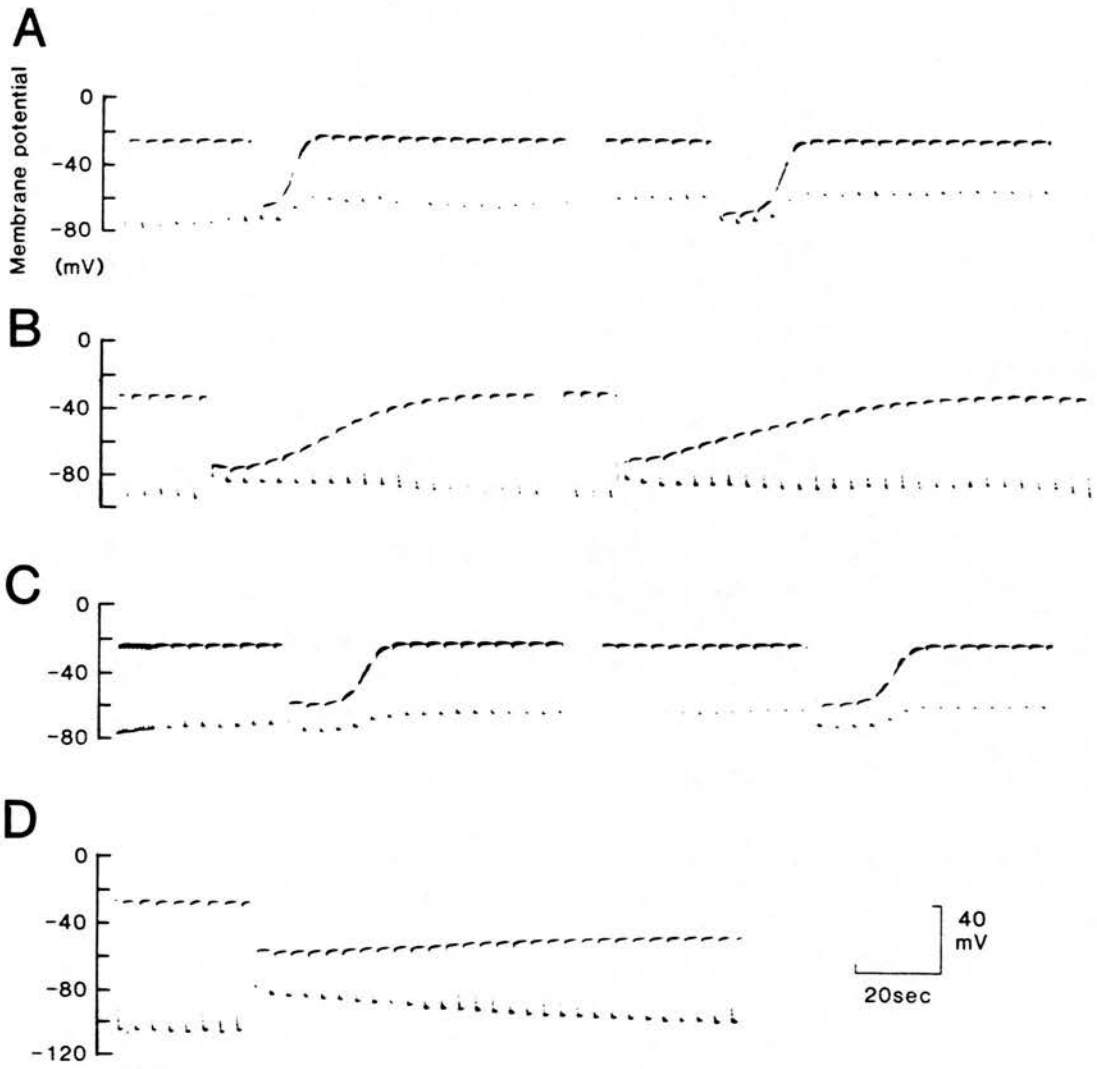

A
Membrane potential
0
-40
-80
(mV)
B
0
-40
-80
C
0
-40
-80
D
0
-40
-80
-120
40
mV
20sec

Table 11A
The effect of lanthanum on various parameters that characterize the calcium-evoked hyperpolarization.

	CONTROL					
Exp.No.	E_r (mV)	$T_{\frac{1}{2}}(V)$ (sec)	$T_{\frac{1}{2}}(R)$ (sec)	$\frac{Ro-Rt}{Ro}$ as %	V (mV)	ΔV (mV)
1	-75	11.0	12.5	81.8	-26	40
	-70	14.0	14.5	82.0	-26	38
2	-69	16.0	18.0	58.0	-18	30
	-78	15.0	21.0	50.0	-18	30
	-78	15.0	16.0	50.0	-18	30
3	-104	26.0	27.0	25.0	-14	22
	-94	26.0	27.0	25.0	-14	20
4	-67	24.0	25.5	60.0	-25	25
	-70	29.0	25.5	62.0	-25	26

Table 11B
The effect of lanthanum on various parameters that characterize the calcium-evoked hyperpolarizations

	LANTHANUM					
Exp.No.	E_r (mV)	$T\frac{1}{2}$(V) (sec)	$T\frac{1}{2}$(R) (sec)	$\frac{Ro-Rt}{Ro}$ as %	V (mV)	ΔV (mV)
1	-76	30.0	32.0	87.5	-33	38
	-77	35.0	35.0	80.0	-33	35
	-72	34.0	38.0	79.0	-30	33
2	-75	90.0	87.0	83.0	-50	22
	-77	110.0	134.0	80.0	-50	22
3	-85	45.0	32.0	48.0	-28	27
	-92	32.0	20.0	32.0	-37	17
4	-70	841*	814*	71.0	-28	32

*See text

Table 11C
The effect of lanthanum on various parameters that characterize the calcium-evoked hyperpolarizations.

	CONTROL					
Exp.No.	E_r (mV)	$T\frac{1}{2}$(V) (sec)	$T\frac{1}{2}$(R) (sec)	$\frac{Ro-Rt}{Ro}$ as %	V (mV)	ΔV (mV)
1	-74	16.0	17.5	65.5	-24	33
	-72	18.0	19.5	74.0	-24	35
2	-75	24.0	26.0	80.0	-31	35
	-67	14.0	16.0	67.0	-28	26
3	-68	7.0	7.0	41.0	-24	17
	-79	7.0	7.0	32.0	-24	17
4	-80	40.0	41.0	61.0	-23	35

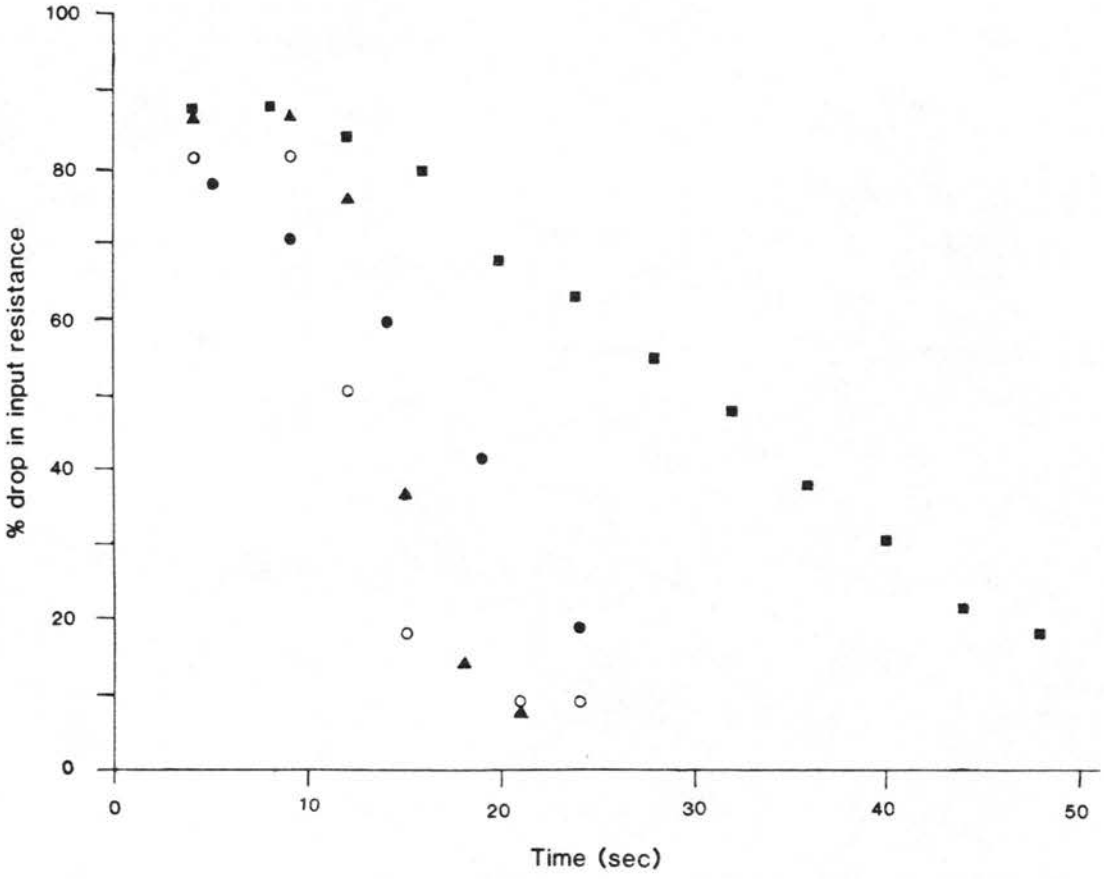
was the greatly retarded rate of recovery of both the membrane potential and the input resistance . This is illustrated in Fig.27. This prolongation of the calcium-induced hyperpolarization was always associated with a corresponding increase in the half time, $T_{1/2}(R)$ (measured as the time taken for (R_o-R_t/R_o) to decrease to 50% of its peak response value) for the transient resistance change . In cells bathed in lanthanum solution, the calcium induced initial rise in membrane input conductance during a hyperpolarization remained at its peak value for longer than it did in normal solutions as the rate at which (R_o-R_t/R_t) recovered was considerably reduced. This is best illustrated in Fig 28 which shows a plot of resistance against time in both control and lanthanum containing media. In one experiment where the cell was bathed in lanthanum (cell 4 in Table 11B) both the membrane potential and the membrane conductance remained at their peak response values and recovery did not occur until lanthanum solution was replaced with normal solution. Values of $T_{1/2}(V)$ and $T_{1/2}(R)$ for the recovery phase of this cell recorded in normal solution are included in Table 11B(*).

Although lanthanum markedly affected the timecourse of the calcium-evoked hyperpolarization, it did not appreciably change the value of the reversal potential (E_r) as shown in Table 11B.

The size ($\triangle V$) of the calcium-evoked hyperpolarization remained unaffected in the presence

Figure 28

A graph of the percentage drop in input resistance $[(R_o-R_t/R_o)x100]$ during a calcium-evoked hyperpolarization as a function of time . The open circles and the filled triangles refer to responses obtained in normal solution prior to lanthanum application. The filled squares refer to the time course of the resistance change during a calcium-evoked hyperpolarization obtained in the presence of 1mM lanthanum. The filled circles refer to the timecourse of the resistance change of a response obtained in normal solution following recovery from lanthanum treatment.


100
80
60
40
20
0
% drop in input resistance
0
10
20
30
40
50
Time (sec)

of external lanthanum in all but one experiment (see results for cell 2 in Table 11B). In cell 2 lanthanum produced a large hyperpolarizing shift of 32mV in the membrane potential. The amlitude of the calcium-induced responses obtained at this more hyperpolarized level was reduced (by 8 to 10 mV) as compared with responses obtained in normal solution (either before or after lanthanum treatment) at more depolarized potentials. This is what would be expected since as the membrane potential approaches the potassium equilibrium potential the electrochemical driving force for potassium ions is reduced.

In all four experiments a consistent increase in the value of (R_o-R_t/R_o) at the peak of the response was observed (see Table 11B). The significance of this result is at present unclear.

Section 5: The effect of high calcium on the calcium-evoked hyperpolarization

Table 12A summarises the results of three experiments where calcium-evoked hyperpolarizations were obtained in normal (4mM) and high (20mM) calcium solutions. In all three experiments the duration $T_{1/2}(V)$ was considerably increased. This was associated with a corresponding increase in $T_{1/2}(R)$. A representative experimental record of the enhancing effect of calcium on the calcium-induced response is shown in Fig.29.

As shown in Table 12A no consistent change in the

Table 12A
The effect of calcium on various parameters that characterise the calcium-evoked hyperpolarization.

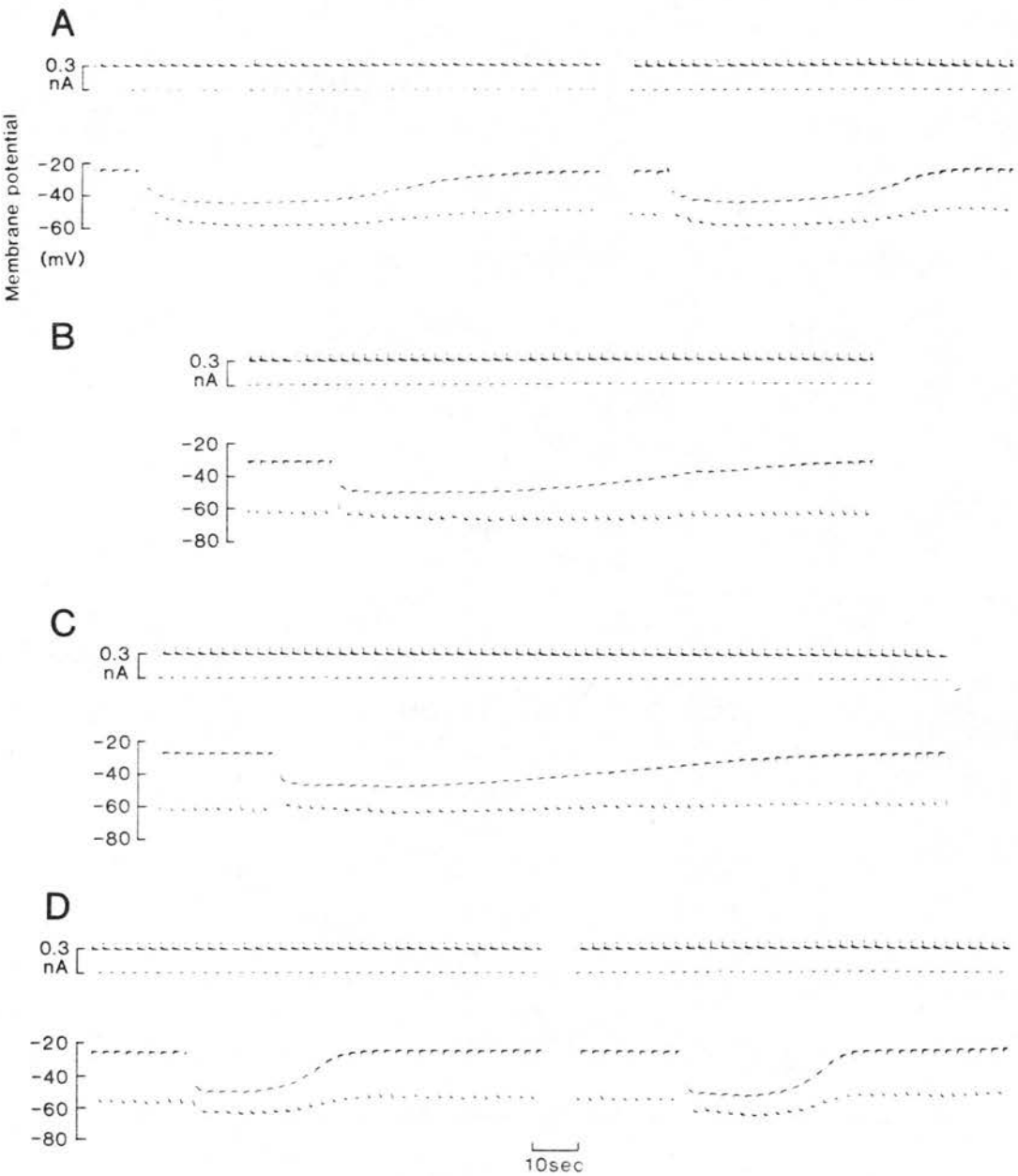
	CONTROL						HIGH CALCIUM					
Experiment No.	E_r (mV)	$T\frac{1}{2}$(V) (Sec)	$T\frac{1}{2}$(R) (sec)	$\frac{Ro-Rt}{Ro}$ %	V (mV)	ΔV (mV)	E_r (mV)	$T\frac{1}{2}$(V) (sec)	$T\frac{1}{2}$(R) (sec)	$\frac{Ro-Rt}{Ro}$ as %	V (mV)	ΔV (mV)
1	-60	42	45	62	-18	26	-45	144	183	73	-15	22
	-67	41	40	50	-20	25						
2	-67	58	53	44	-23	19	-69	70	60	54	-30	19
	-62	47	49	50	-23	19	-65	70	83	57	-28	21
3	-76	15	20	53	-36	31	-71	42	47	65	-37	22
							-79	27	27	58	-38	24

Table 12B
The effect of calcium on various parameters that characterise the calcium-evoked hyperpolarization.

	RECOVERY					
	E_r	$T_{\frac{1}{2}}(V)$	$T_{\frac{1}{2}}(R)$	$\frac{Ro-Rt}{Ro}$	V	ΔV
Experiment No.	(mV)	(sec)	(sec)	as %	(mV)	(mV)
1	-68	23	27	67.0	-11	38
	-77	22	23	47.0	-11	31
2	-67	24	27	59.0	-25	25
	-70	29	31	57.0	-25	26
3	-70	20	21	76.0	-38	24
	-73	22	24	81.0	-34	32

Figure 29

The effect of alterations in external calcium on the profile of the calcium-evoked hyperpolarization. Responses obtained in normal solution containing 4mM calcium are presented in A. B and C show responses obtained in a solution containing 20mM calcium. Recovery from exposure to 20mM external calcium and return to a solution containing 4mM calcium is shown in D. Identical constant current pulses (placed above the voltage traces) were passed via the recording electrode throughout the course of the experiment. Input resistance is indicated by the size of the resulting electrotonic potentials (shown as downward deflections on the voltage traces).


A
0.3
nA
-20
-40
-60
(mV)
Membrane potential
B
0.3
nA
-20
-40
-60
-80
C
0.3
nA
-20
-40
-60
-80
D
0.3
nA
-20
-40
-60
-80
10sec

reversal potential (E_r) was found between responses obtained in normal and high calcium solutions. The augmenting effect of high external calcium on the timecourse of both potential and resistance was found to be fully reversible (see Table 12B).

Section 6: The effect of high external barium on the calcium- evoked hyperpolarization

The effect of high external barium was also investigated in three experiments. The amplitude of the calcium- evoked hyperpolarization was greatly reduced (see Table 13A). An experimental record of one of these experiments is shown in Fig.30.

In all three cases barium considerably reduced the reversal potential of the response and the initial % loss in resistance (R_o-R_t/R_o) was also considerably reduced. The timecourse of the potential change $T_{1/2}(V)$ did not appreciably change under high barium conditions. Partial but not complete recovery of the calcium-evoked response was observed after the eggs had been returned to normal solution as shown in Fig 30C. A summary of the results obtained after recovery from exposure to external barium is presented in Table 13B.

Section 7: The effect of high magnesium on the calcium-evoked hyperpolarization

Elevation of external magnesium from 1.7mM to 20 mM (n=4) and to 11mM (n=1) had no effect on any of the calcium-evoked response characteristics. A summary of

Table 13A
The effect of barium on various parameters that characterize the calcium-evoked hyperpolarization

	CONTROL					BARIUM				
Experiment No.	E_r (mV)	$T_{\frac{1}{2}}(V)$ (sec)	V (mV)	ΔV (mV)	$\frac{Ro-Rt}{Ro}$ as %	E_r (mV)	$T_{\frac{1}{2}}(V)$ (sec)	V (mV)	ΔV (mV)	$\frac{Ro-Rt}{Ro}$ as %
1	-90	22	-25	59	90	-66	29	-27	35.0	85
	-90	26	-25	59	91	-78	20	-27	43.5	65
	-80	33	-28	59	92	-49	25	-27	14.0	61
						-81	36	-27	24.0	55
						-58	38	-27	9.0	30
2	-81	40	-23	45	60	-53	26	-18	17	32
						-52	33	-19	15	42
3	-90	11	-35	19	34	-65	12	-37	6	19
	-86	11	-35	21	41	-70	-	-37	6	25
	-84	12	-35	21	48	-51	17	-36	6	41

Table 13B

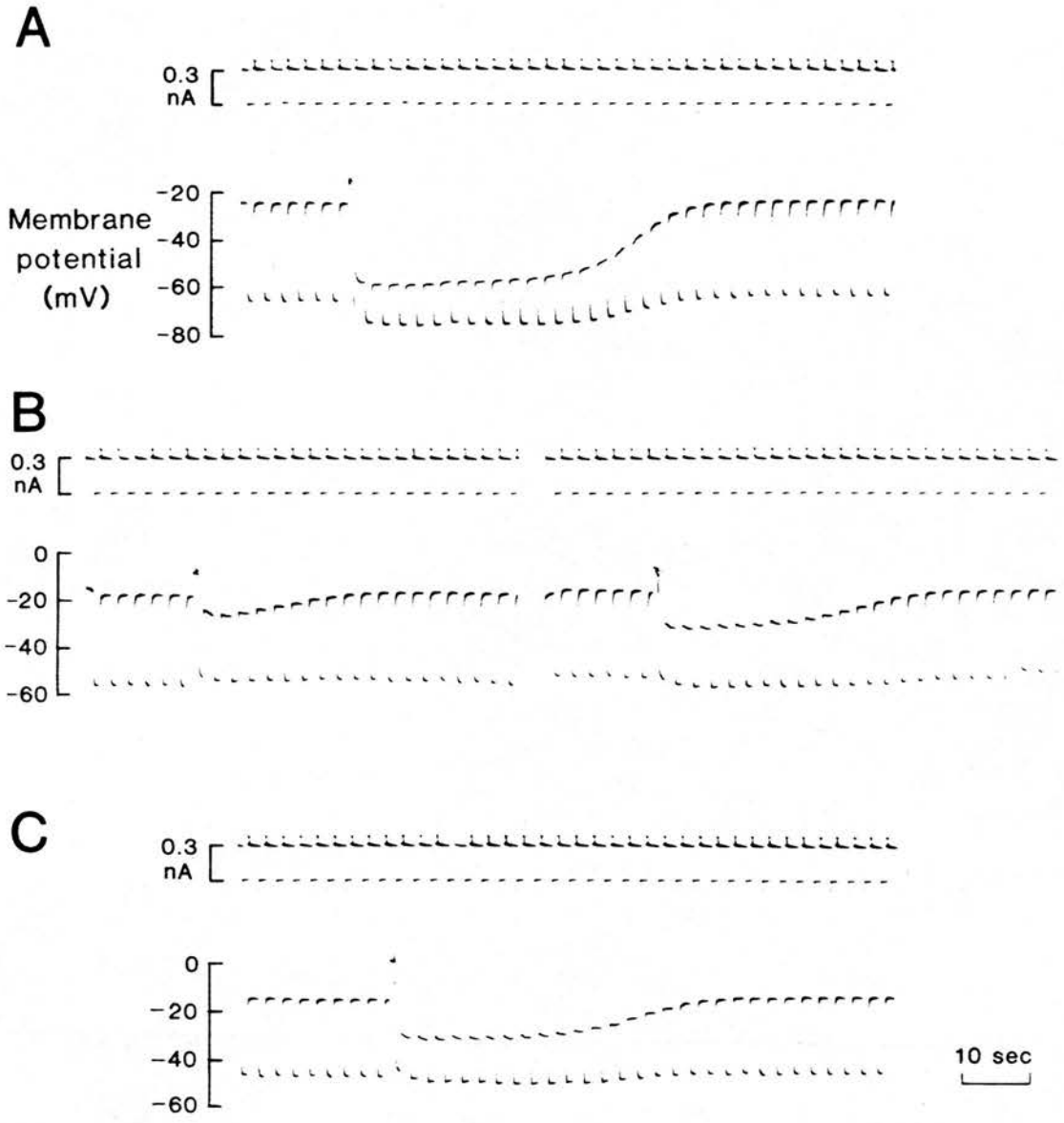
The effect of barium on various parameters that characterize the calcium-evoked hyperpolarization.

	CONTROL RECOVERY				
Experiment No.	E_r (mV)	$T\frac{1}{2}$(V) (sec)	V (mV)	ΔV (mV)	$\frac{Ro-Rt}{Ro}$ as %
1	-	-	-	-	-
2	-56	33	-17	18	48
	-62	45	-17	20	44
3	-70	20	-36	25	74
	-70	22	-34	27	74

Figure 30

The blocking effect of external barium on the calcium-evoked hyperpolarization. A shows a calcium-induced response obtained in normal solution whereas in B calcium injections were performed in the presence of 20mM barium. Notice the greatly diminished size of the responses obtained in the barium containing solutions as compared to the responses obtained in normal solution. Notice also that the loss in input resistance as indicated by the difference in the amplitudes of the electrotonic potentials prior and at the peak of the response is substiantially reduced in responses obtained in high barium solutions. C shows a response obtained after recovery from exposure to barium.

Constant current pulses of identical strengh were used throughout the experiment .


A
0.3
nA
Membrane
potential
(mV)
-20
-40
-60
-80
B
0.3
nA
0
-20
-40
-60
C
0.3
nA
0
-20
-40
-60
10 sec

these results is given in Table 14.

Section 8: The effect of dinitrophenol (DNP) on the membrane potential and resistance

External application of DNP at a concentration ranging between 0.2 to 2mM produced membrane depolarization which was always associated with a substantial fall in input resistance (mean reduction ± SD=36±18%,n=6). A representative record of the effect of DNP on potential and resistance is shown in Fig.31A. Sometimes partial or complete recovery from exposure to DNP could be achieved by bathing eggs in DNP-free normal Ringer solution (see Fig.31B). The depolarizing action of DNP is probably related to its ability to transport H^+ across cell membranes (Boron & de Weer, 1976 ; see review by Mclaughlin & Dilger,1980)

Section 9: The effect of DNP on the calcium-evoked response

Calcium injections in the presence of 0.2 to 2mM DNP produced a hyperpolarization with considerably longer duration than responses obtained in normal solution (see Table 15A). Unlike lanthanum , DNP extended the duration of the response by markedly prolonging the plateau phase as illustrated in Fig.32B & C. Neither the reversal potential of the response parameters nor any of the other response parameters were appreciably affected by DNP. The results of the recovery of the response from treatment with DNP are shown in Table 15B.

Table 14
The effect of magnesium on various parameters that characterise the calcium-evoked hyperpolarizations.

	NORMAL SOLUTION						MAGNESIUM SOLUTION					
Experiment No.	E_r (mV)	$T_{\frac{1}{2}}$(V) (sec)	$T_{\frac{1}{2}}$(R) (sec)	V (mV)	ΔV (mV)	$\frac{Ro-Rt}{Ro}$ as %	E_r (mV)	$T_{\frac{1}{2}}$(V) (sec)	$T_{\frac{1}{2}}$(R) (sec)	V (mV)	ΔV (mV)	$\frac{Ro-Rt}{Ro}$ as %
1	-85	18.0	17.0	-37	31	65.0	-80	14.0	17.0	-34	25.5	55.0
	-86	17.0	19.5	-37	31	64.0	-78	14.0	16.5	-34	24.5	55.5
2	-75	28.0	30.0	-75	22.5	37.5	-71	30.0	32.0	-15	17.5	31.0
	-83	28.0	25.0	-15	22.5	31.0	-62	26.0	30.0	-13	15.0	31.0
3	-92	17.o	18.5	-47	29	64.0	-86	17.0	18.5	-45	24.5	56.0
	-90	17.0	20.5	-46	27	60.0	-90	17.0	21.0	-43	27.0	57.0
4	-77	19.0	22.0	-18	44	74.0	-60	12.0	17.5	-14	31	77.0
	-78	17.0	20.0	-19	42	71.0						
5	-67	24.0	28.o	-25	25	57.0	-62	26.0	27.0	-22	24	60.0
	-70	29.0	32.0	-25	26	57.0	-62	24.0	26.0	-20	26	60.0

Figure 31

The depolarizing effect of dinitrophenol (DNP) on the membrane potential of hamster eggs. A shows that external application of 2mM DNP indicated by the arrow caused a reduction in both the membrane potential and the input resistance of the egg. Repolarization of the membrane potential and an increase in the input resistance occurred after the DNP was washed off as shown in B. In B the arrow indicates the time of application of normal DNP free solution.

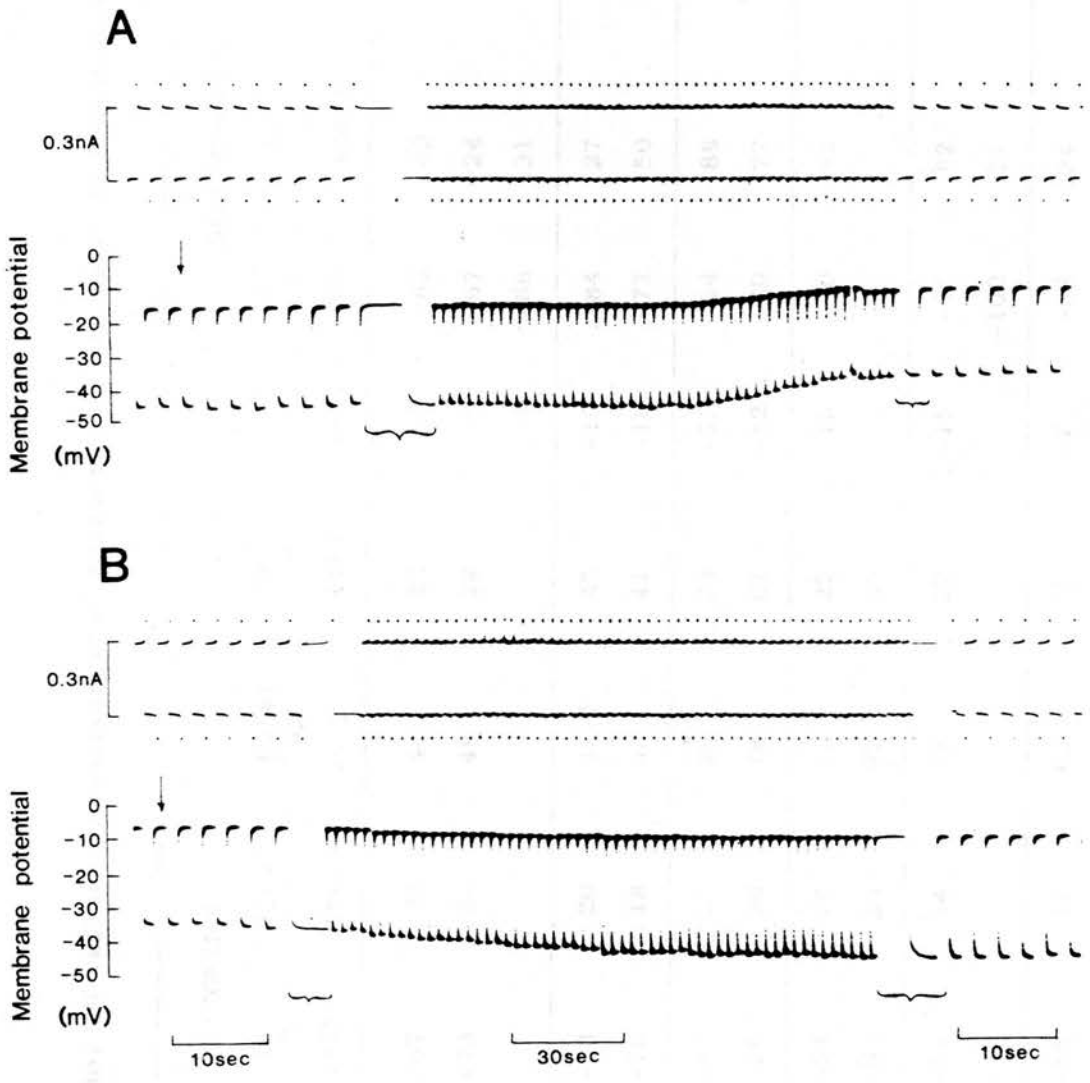

A
0.3nA
Membrane potential
0
-10
-20
-30
-40
-50
(mV)
B
0.3nA
Membrane potential
0
-10
-20
-30
-40
-50
(mV)
10sec
30sec
10sec

Table 15 B

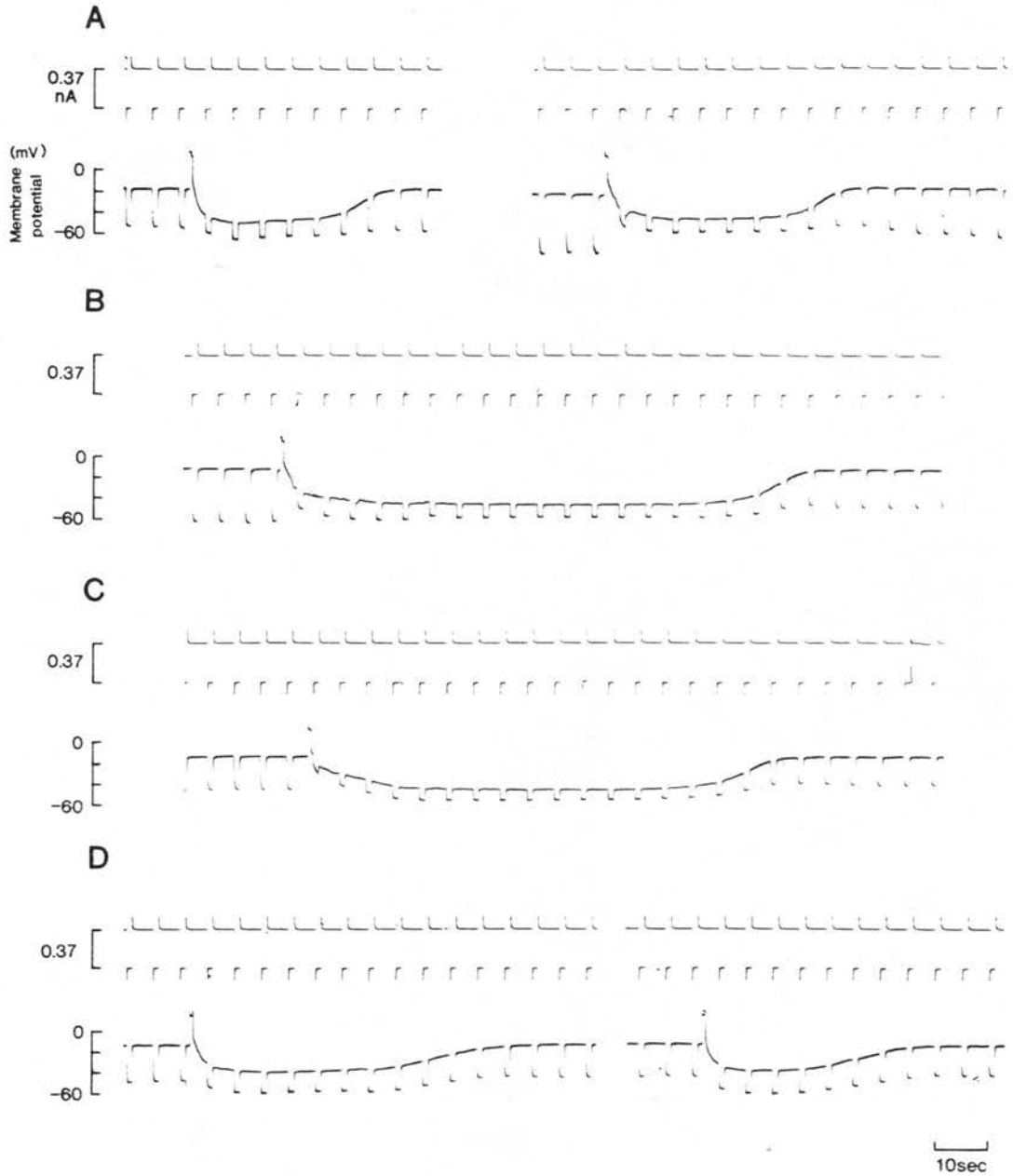
The effect of dinitrophenol on various parameters that characterize the calcium-evoked hyperpolarization.

	CONTROL	RECOVERY			
Experiment No.	V (mV)	E_r (mV)	$T_{\frac{1}{2}}(V)$ (sec)	$\frac{Ro-Rt}{Ro}$ as %	ΔV (mV)
1	-10	-67	30	36	30
	-9	-76	33	44	30
2	-	-	-	-	-
3	-15	-83	43	41	28
4	-	-	-	-	-
5	-21	-51	12	43	15
	-21	-69	14	46	22
	-19	-71	10	29	15
6	-	-	-	-	-

Figure 32

The prolonging effect of dinitrophenol (DNP) on the duration of the calcium-evoked hyperpolarization. Responses obtained in control solutions are shown in A whereas responses obtained in the presence of 0.2mM DNP are shown in B and C. Recovery from DNP application is shown in D.

Notice the prolonged reduction in the input resistance that accompanies the hyperpolarizing response.


A
0.37
nA
(mV)
Membrane potential
0
-60
B
0.37
0
-60
C
0.37
0
-60
D
0.37
0
-60
10sec

Section 10: The effect of caffeine on the calcium-evoked response

Caffeine (10mM) produced no detectable effect on either the passive membrane parameters or the calcium-evoked hyperpolarization (n=2).

DISCUSSION

The good correlation between reversal potential and the recovery time of the membrane conductance indicates that the membrane almost always suffered some degree of temporary breakdown during the course of an ionophoretic calcium pulse. The magnitude of the leak conductance during such a pulse can be estimated from experiments where divalent ions other than calcium or strontium were injected. Considering for example the case shown in Fig.24 where barium was injected, the input resistance fell from 115MΩ ($g = 8.7 \times 10^{-9}$S) to 58MΩ($g_p = 1.7 \times 10^{-8}$S) at the peak of the depolarization (-16mV). Hence $g_{il} = g_p - g = (1.7-0.87) \times 10^{-8}$S. Analysis of a number of similar records and others where it was suspected that calcium injections also produced such breakdown it was found that the value of the leak conductance pathway generated during large depolarizing current pulses fell within the range $0.6-1.65 \times 10^{-8}$ S.

Calcium-evoked responses with short time constants (T_R<10sec,n=10responses;see Table 9) for the recovery of the % loss in input conductance were probably not seriously contaminated from g_{il}. The mean(±SD) value of the reversal potential (E_r) for these responses was -77±7mV. This value probably represents a more reliable estimate of E_r than the mean value(±SD) of -69±11mV derived from the total population of the results (see Table 7 in Part II of chapter II). Moreover , the former estimate is in closer agreement with the value of -82.7±2.2mV(Mean±SD,n=34) reported by

Miyazaki and Igusa (1982). For these responses, g_K, calculated from the conductance data was $2.1 \pm 1.4 \times 10^{-8}$S (Mean$\pm$SD)

The timecourse of the potential change in responses with low reversal potentials can be described quantitatively by taking into account g_{il}. Consider, for example, response C of Fig.22. The value of the potential at the peak of the hyperpolarization (V_p) was -42mV. Prior to injection the membrane potential (V) was -23mV and the input conductance (g) was 5.0×10^{-9} S. Given that E_K=-80mV and that $g_K = 2.0 \times 10^{-8}$ S the value of the membrane potential at the peak of the response (V_p) can be predicted by assuming an injection-induced leak battery (E_{il})=-9mV , $g_{il} = 1.0 \times 10^{-8}$S (range 0.6 to 1.65×10^{-8}S) and using the following equation:

$$V_p = \frac{E_K g_K + V g + E_{il} g_{il}}{g_K + g + g_{il}}$$

$$= \frac{(2.0 \times 10^{-8} x - 0.08) + (5.0 \times 10^{-9} x - 0.023) + (1.0 \times 10^{-8} x - 0.009)}{(1.8 + 1.125 + 0.5) \times 10^{-8}}$$

$$= -40\text{mV}$$

Thus the predicted value is in very good agreement with the observed value of -42mV. The value of V at the peak of the subsequent depolarizing phase (-15mV) can also be accounted for by assuming that g_K declines to zero and $g_{il} = 1.0 \times 10^{-8}$S (calculated value of V_p in the above example equals -14mV). The timecourse of the membrane potential for response A of Fig.22 can also be described quantitatively by similar considerations.

For three calcium-induced responses with reversal

potentials of -62, -64 and -58mV, plots of (R_o-R_t/R_o) against time were drawn and two exponential components could be distinguished. After exponential curve peeling a fast time constant for the recovery of the resistance was extracted with values of 4.5,5 and 6sec. This value is in good agreement with the recovery time constants of responses with high reversal potentials close to the expected E_K (see page 72), and probably represents the rate at which K^+-channels inactivate. The corresponding time constant for the second exponential (slow time constant) prior to curve peeling had values of 118,73 and 124sec. Two exponents , a fast one and a slow one for the recovery of the membrane resistance of calcium-evoked hyperpolarizations have been reported by Meech(1974a) following pressure injections of calcium chloride into Helix neurons. However, no definite conclusion was reached as to what the two exponents represented. The interpretation offered here is that they represent membrane recovery from breakdown induced during ionophoretic pulses. This is supported by the observations of Zimmermann, Vienken and Pilwat (1984) who have found that for biological membranes the resealing times following breakdown by high voltage pulses (field stimulation) was of the order of minutes.

The hyperpolarizing effect of external lanthanum and the parallel increase in the cell's input resistance may indicate an improved seal between the tip of the microelectrode and the plasma membrane. A similar enhancing effect of lanthanum on the membrane

potential has been observed in Ehrlich ascites tumor cells (Smith, Mikiten & Levinson, 1974). In these cells the increase in membrane potential was maximal at an external lanthanum concentration of 1mM, the membrane potential being shifted from a value of -17.8±1.7 to a value of -56.0±4.1mV (mean±SD). In a subsequent study Levinson, Mikiten and Smith (1972) reported that lanthanum exerted a concentration-dependent effect on the electrolyte content of tumor cells. They found that application of 1mM external lanthanum reduced cellular K^+, Cl^- and Na^+ content by 87%, 79% and 21% respectively. The loss in Na^+ occurred even against its electrochemical gradient. These workers were not able to account for the observed value of the membrane potential in the presence of external lanthanum in spite of knowing the concurrent changes in the internal ionic composition of the cells. They therefore suggested that in addition to possible permeability changes electrogenic ion transport might have been involved. As suggested above another possibility is that lanthanum might effect a steady membrane hyperpolarization by increasing the seal resistance between the microelectrode and the egg plasma membrane. Evidence, however, exists from preliminary experiments (Bountra, Martin & House, unpublished) suggests that lanthanum facilitates giga-seal formation between the egg membrane and patch-micropipettes.

The extended timecourse of the calcium-induced

hyperpolarization in lanthanum solutions probably indicates a disturbance of cell calcium homeostasis. The action of lanthanum is most likely confined to the outer surface of the egg's plasma membrane, where it probably blocks a calcium-ATPase. Support for this hypothesis comes from the work of Sarkadi, Schubert and Gardos (1978) who have shown that in fresh human red cells that lanthanum is (a) impermeant, (b) completely blocks active calcium-efflux and (c) inhibits Ca^{2+}-Mg^{2+}-ATPase when applied at 0.25mM. The possibility that lanthanum might be inhibiting a Na^{+}-Ca^{2+} exchange system cannot be excluded although Kutzung, Reuter & Porzig (1973) have shown that lanthanum does not inhibit Na^{+}-Ca^{2+} exchange in cardiac muscle. The observation that high external calcium mimicks this action of lanthanum , albeit to a lesser extent coupled with the fact that lanthanum competes with calcium for binding sites either on the cell surface or intracellularly (see review by Martin & Richardson, 1979) suggests that they might have a similar mode of action.

The prolonging effect of high calcium on the duration of the calcium-evoked hyperpolarization has also been observed in the after-hyperpolarizing response of Leech neurones (Jansen & Nicholls, 1973). Elevation of external calcium from 1.8 to 10 mM in these neurones, markedly enhanced both the amplitude and duration of the hyperpolarization seen in these cells following a brief train of impulses. This led

these authors to suggest that calcium produced this effect because it could enter these neurones during a train of action potentials presumably through voltage gated calcium channels. However, this cannot be the explanation for our results since in all cases the membrane potential prior to injecting calcium was well below the threshold for activating voltage-gated calcium channels known to be present in hamster eggs (Miyazaki & Igusa, 1982).

It could be argued that the large depolarizing excursions (+ 150mV from the resting potential) produced by the ionophoretic calcium pulse could bring about temporary electrical breakdown of the egg's plasma membrane, thus allowing additional calcium influx into the cell. Admittedly some degree of breakdown does occur as a result of such pulses. However if calcium could readily enter the cell during large depolarizations, then we would expect to see hyperpolarizing responses by depolarizing current pulses through pipettes containing divalent ions other than calcium or strontium. This was not observed which indicates that depolarizing pulses per se cannot account for any significant calcium influx into the cell.

The augmenting effect of DNP on the duration of the hyperpolarization further implicates the involvement of an active calcium-uptake and/or extrusion mechanism operating during the recovery phase of the response. Alternatively the prolongation of the calcium-evoked

response might be due to a direct effect of a fall in intracellular pH brought about by DNP on the inactivation kinetics of the Ca^{2+}-activated K^{+}-channels.

The effect of DNP on the passive electrical properties of hamster eggs was to produce a depolarization associated with a concomitant reduction input resistance. DNP has also been found to produce a drop in the input resistance of molluscan neurones Carpenter, Snover & Barker,1971 ; Meech,1974) and of cortical neurones (Krnjevic, Godfraind, Pumain & Provini,1970). In molluscan neurones and in cortical neurones,however, the above workers have reported that the drop in resistance was associated with a hyperpolarization of the cell membrane. The general interpretation proposed for the membrane hyperpolarization was that it resulted from changes in membrane permeability which were in turn caused by a DNP-induced steady rise in intracellular free calcium. In hamster eggs the reduction in input resistance observed in the presence of DNP was accompanied by a depolarization which suggests that the mode of action of DNP was confined to the transfer of H^{+} across the egg plasma membrane (Boron & De Weer, 1976 ;see review by Mclaughlin & Dilger,1980).

The inability of high magnesium solutions to prolong the duration of the response argues against the possibility that the actions of lanthanum or high calcium are mediated through a non-specific screening

effect , which is known to be exerted by polyvalent ions on membrane surface charges (Hille,1968).

Externally applied barium is known to block Ca^{2+}-activated K^{+}-channels in molluscan neurones (Eckert & Lux,1977 ; Connors 1979) and pig pancreatic acinar cells (Iwatsuki,1984). The results presented indicate a similar action by barium on Ca^{2+}-activated K^{+}-channels in hamster eggs.

In conclusion these experiments show that lanthanum, high calcium and DNP considerably delay the recovery phase of the calcium-evoked hyperpolarization. Localization of the site of action of these agents would help provide important clues about the nature of intracellular calcium regulation in mammalian eggs.

CHAPTER IV

THE BLOCKING EFFECT OF CHOLINE CHLORIDE AND TETRAETHYLAMMONIUM (TEA) ON CALCIUM-ACTIVATED POTASSIUM CHANNELS IN HAMSTER EGGS

METHODS

The methods used for obtaining, treating and recording from hamster eggs were identical to those described in the previous paper. The calcium injections were also performed as described in Chapter I.

The solutions used for this series of experiments are indicated in Table 5 of Chapter I

RESULTS

Section 1: The effect of choline chloride on the membrane potential and input resistance

Replacement of external sodium chloride with choline chloride (see Tables 16A,16B & 16C) was almost always associated with a steady hyperpolarizing shift of about 5mV (difference between mean($\pm$SD) of controls, -22$\pm$7mV and choline containing media, -27$\pm$14mV). A corresponding rise of the cell's input resistance (by about 25% of its control value) also occurred as a result of this ionic substitution. This is what would be expected if the cell membrane were more permeable to sodium ions than to choline ions.

In three out of four experiments where lithium was used as a substitute for sodium a slight hyperpolarization occurred (maximum of 4mV, see Tables 17A , 17B & 17C) associated with a rise in the input

Table 16 A

The effect of substituting choline for sodium on various parameters that characterize the calcium-evoked hyperpolarization .

	CONTROL				
Experiment No.	E_r (mV)	$T_{\frac{1}{2}}$(V) (sec)	$\frac{Ro-Rt}{Ro}$ as %	V (mV)	ΔV (mV)
1	-65	10	71	-27	31
2	-60	27	69	-20	27
3	-86	16	67	-16	46
4	-50	21	67	-19	21
5	-85.	25	35	-16	25
6	-79	20	68	-22	39
7	-54	25	75	-18	25
8	-92	14	85	-37	47
9	-66	47	88	-23	38
10	-105	20	30	-12	29
11	-73	18	80	-29	35
12	-58	25	65	-15	30
13	-66	20	78	-26	34
14	-70	25	74	-30	30
mean ±SD	-72±16	22±9	44±27	- 22±7	33±8

Table 16 B

The effect of susbstituting choline for sodium on various parameters that characterize the calcium-evoked hyperpolarization.

	SODIUM-FREE (CHOLINE)				
Experiment No.	E_r (mV)	$T_{\frac{1}{2}}$(V) (sec)	$\frac{Ro-Rt}{Ro}$ as %	V (mV)	ΔV (mV)
1	-74	13	44	-24*	22
2	-47	17	54	-20*	14
3	-58	-	31	-38	5
4	-35	11	41	-24*	6
5	-86	16	25	-30	14
6	-74	17	39	-36	14
7	-51	23	52	-25	14
8	-76	13	68	-45	22
9	-39	14	72	-23*	10
10	-62	16	52	-20	22
11	-75	14	26	-34	9
12	-32	20	62	-18	9
13	-44	17	80	-29	12
14	-39	24	47	-9	12
Mean ±SD	-57±18	17±4	49±17	27±9	13±6

Table 16 C

The effect of substituting choline for sodium on various parameters that characterize the calcium-evoked hyperpolarization.

	CONTROL (RECOVERY)				
Experiment No.	E_r (mV)	$T_{\frac{1}{2}}$ (V) (sec)	$\frac{Ro-Rt}{Ro}$ as %	V (mV)	ΔV (mV)
1	-76	16	56	-25	29
2	-78	16	27	-20	15
3	—	—	—	—	—
4	-59	25	55	-23	20
5	-75	16	72	-20	40
6	-78	20	34	-13	22
7	-65	22	40	-14	20
8	-	-	-	-	-
9	-58	16	85	-22	30
10	-57	15	69	-17	32
11	-76	15	53	-36	31
12	-66	19	47	-6	28
13	-72	18	61	-22	32
14	-47	20	46	-4	20
Mean±SD	-67±10	18±3	54±16	-18.5±9	27±7

Table 17 A

The effect of substituting lithium for sodium on various parameters that characterize the calcium evoked hyperpolarization.

	NORMAL				
Experiment No.	E_r (mV)	$T_{\frac{1}{2}}$(V) (sec)	$\frac{Ro-Rt}{Ro}$ as %	V (mV)	ΔV (mV)
1	-79.5	26.8	67	-12	45
	-82	28.0	75	-12.5	43.5
2	-64.5	27	54.5	-15	27
	-59	29	61.5	-15	27
	-64	29	58.0	-14	29
3	-55	35	61.5	-15	26
	-60	35	62.5	-14	29
4	-82	32	33	-13	23
	-75	37	37	-13	23

Table 17 B

The effect of substituting lithium for sodium on various parameters that characterise the calcium-evoked hyperpolarization .

	LITHIUM				
	E_r	$T\frac{1}{2}$(V)	$\frac{Ro-Rt}{Ro}$	V	ΔV
Experiment No.	(mV)	(sec)	as %	(mV)	(mV)
1	-61.5	24.4	85	-16	39
	-68.0	75.2	89	-8	54
	-70.0	46.4	88	-8	55
2	-57	22	61.5	-16	25
	-60	23	64.0	-14	28
	-52	24	54.5	-8	28
3	-49	31	57	-16*	19
	-60	25	46	-16*	20.5
	-59	27	53	-16*	23
	-60.5	29	46	-15*	21
4	-51	27	50	-15	23
	-93	18	32	-14	25
	-78	27	40	-13	26

*Current clamped at this value

Table 17 C

The effect of substituting lithium for sodium on various parameters that characterize the calcium evoked hyperpolarization.

CHOLINE CHLORIDE

Experiment No.	E_r (mV)	$T_{\frac{1}{2}}$(V) (sec)	$\frac{Ro-Rt}{Ro}$ as %	V (mV)	ΔV (mV)
1	-66	30.4	65	-25	27
	-76	29.2	58	-26	29
	-72	37	60	-10*	37
	-67	19.2	61.5	-10*	35
2	-41	17	43	-15	11
	-24.5	18	45	-8	7.5
3	-57	47	43	-15	18
	-49	-	40	-18	12.5
	-66	52	27	-15	14

*Current clamped at this value.

resistance from 119±16MΩ to 125±13MΩ, that is a percentage change of only 5%. However, in one experiment a depolarization of 10mV was observed instead accompanied by a fall in input resistance from 132MΩ in sodium containing solution to 40MΩ in lithium solution.

Upon substitution of choline with lithium an additional large increase in the membrane input resistance was observed (by as much as 75% in 2 experiments).

Section 2: The suppressing effect of choline chloride on the calcium-evoked hyperpolarization

In all experiments where choline chloride was used as a substitute for sodium chloride the amplitude (ΔV) of the calcium-evoked hyperpolarization was consistently reduced (see Tables 16A, 16B & 16C). However, since the membrane potential tended to hyperpolarize in the presence of choline chloride, it might be argued that the reduced amplitude of the calcium-induced response could have been caused by the decrease in the electrochemical driving force (E_m-E_k) for potassium ions. Although this might be a contributing factor in obtaining diminished responses, it cannot account for the reduced responses obtained in choline media where the hyperpolarizing shift in membrane potential did not exceed 5mV (results marked with an asterisk on Table 16B)

It is clear from the results of Tables 16A and 16B that the percentage loss in membrane input resistance

$(R_o - R_t)/R_o$ measured at the peak of the calcium-evoked hyperpolarizing response was consistently supressed in choline chloride solutions relative to sodium containing solutions. This is illustrated in Fig.33

The size of the electrotonic potentials at rest (P), and during the response (p), for any given current pulse I are given by

$$P = I / g_K \ldots(1) \text{ and } p = I / g+g_K \ldots(2)$$

where g is the conductance at rest and

g_K is the conductance added during the response as a result of Ca^{2+}-activated K^+-channel opening.

It follows from equation (2) that for $g_K >> g$ p I/ g_k and the ratio $(R_o-R_t)/R_o$ would assume relatively large values. However, if g_K is partially blocked then the amplitude of p would tend to approach that of P and hence the value of R_o-R_t/R_o would decrease.

In order to check that the suppression of $(R_o-R_t)/R_o$ was not caused by a redistribution of intracellular sodium when external sodium was removed separate experiments were carried out where lithium was used to replace sodium. In three such experiments no appreciable difference was observed between responses obtained in normal solutions and responses obtained in lithium containing solutions. The lithium was then replaced with choline chloride and hyperpolarizations in response to calcium injections were elicited (see Fig 34). In order to get a better comparison between responses in different media, the cells were current

Figure 33

The blocking effect of choline chloride on the calcium-evoked hyperpolarization. A shows a hyperpolarizing response obtained in normal solution whereas B shows a response obtained in a solution where choline was substituted for sodium. Note that both the amplitude of the hyperpolarization and the loss of input resistance (i.e., increase in input conductance) at the peak of the response were considerably reduced. C shows recovery from exposure to choline.

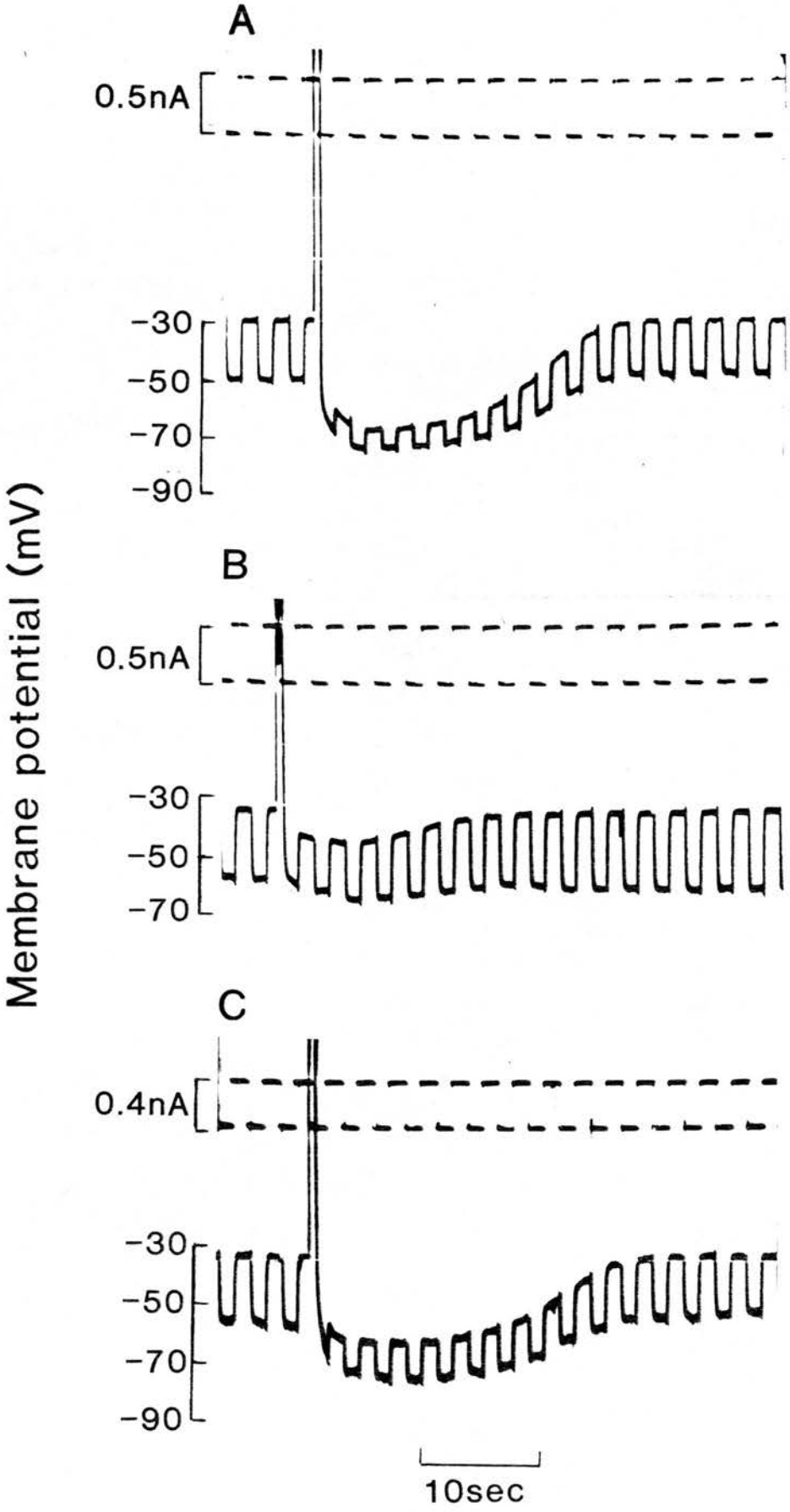
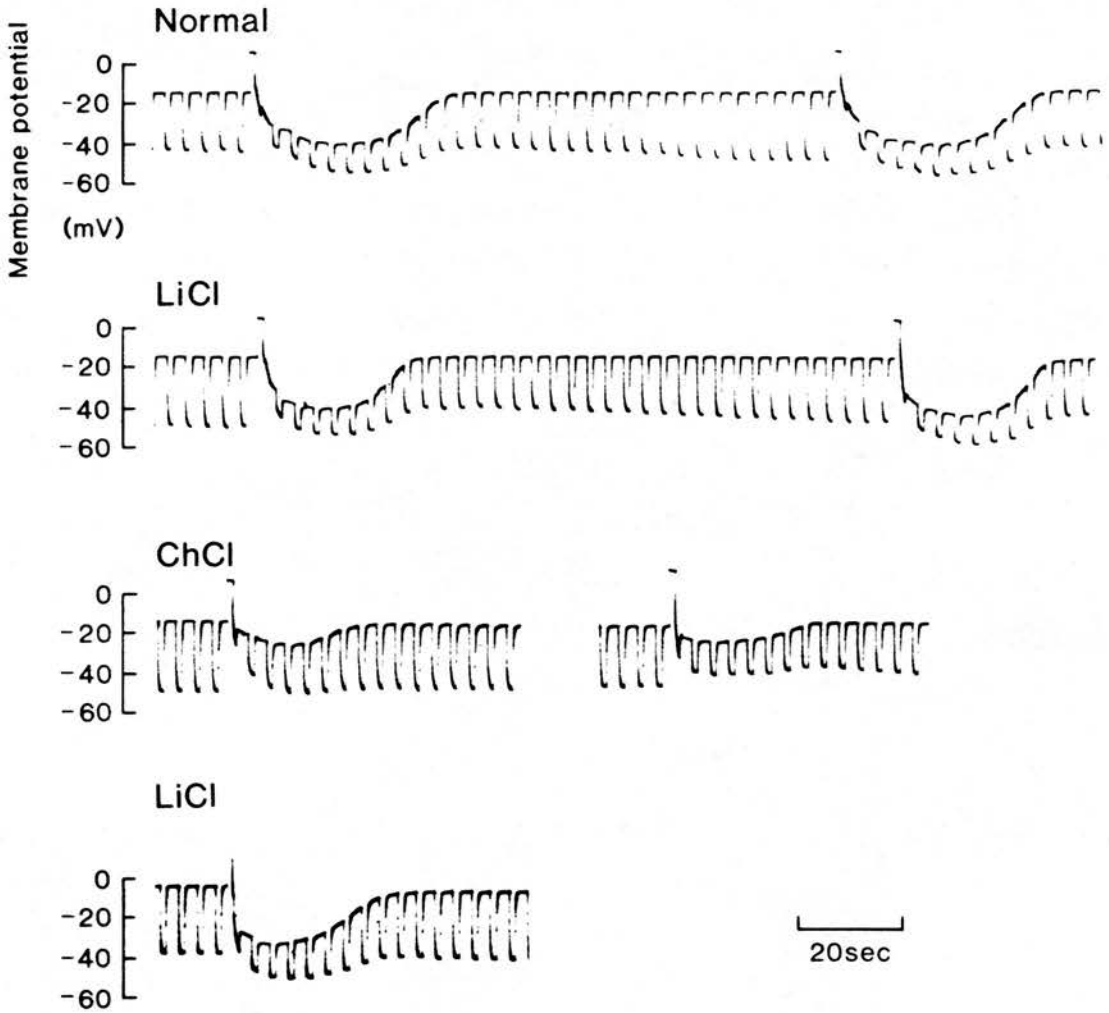

A
0.5nA
-30
-50
-70
-90
B
0.5nA
-30
-50
-70
C
0.4nA
-30
-50
-70
-90
Membrane potential (mV)
10sec

Figure 34

The effect of different solution substitutions on the calcium-evoked response profile. All responses were obtained from the same egg. Note that the amplitude of the response is greatly reduced when choline is substituted for sodium. Notice also that the loss in the input resistance at the peak of the responses is markedly suppressed in the presence of external choline as compared to the drop in input resistance that occurs in either normal or lithium containing solution. The experiments were carried out in the sequence shown in the figure, starting in normal solution and ending in a lithium containing solution.


Membrane potential
(mV)
Normal
LiCl
ChCl
LiCl
0
-20
-40
-60
20sec

clamped where necessary to approximately the same potential during calcium-injections. The results of these experiments are shown in Tables 17A, 17B and 17C. An asterisk next to the V values of this table signifies that the cell membrane was current clamped at that potential). It is evident from these results that it is not the replacement of sodium per se which is responsible for the marked reduction in response amplitude and the fall in $(R_o-R_t)/R_o$ since responses obtained in lithium chloride solutions resembled closely those obtained in normal media. However, in the presence of choline chloride the peak amplitude (ΔV) of the calcium-induced response and also the fall in $(R_o-R_t)/R_o$ at the peak of the response were markedly reduced. These experiments demonstrate further that choline tends to block Ca^{2+}-activated K^+-channels.

Analysis of variance for the reversal potential showed a significant difference ($p=0.05, n=14$) between responses obtained in choline containing media and responses obtained in control solutions. Although the apparent blocking effect exerted by choline can be explained in terms of its molecular dimensions, its effect on the reversal potential cannot be easily understood. A possible reason for this result might be due to the fact that the determination of the reversal potential of partially blocked responses is subject to greater error than the determination of the reversal potential of unblocked responses. This is because in partially blocked responses the value of p (ie the size

of the electrotonic potential at the peak of the response) gets closer to the value of P (the size of the electrotonic potential at rest) and hence a small error in the determination of p will produce a comparatively large error in the calculated value of the reversal potential (E_r).

Section 3: The suppressing effect of tetraethylammonium (TEA) on the calcium-evoked hyperpolarization

Application of external TEA (at either 10 or 20mM) had no observable effect on either the resting potential or the input resistance.

However, TEA at a concentration of 20mM brought about a decrease in the calcium-induced hyperpolarization very similar to the that produced by choline. A representative example of such a blocking action by external TEA is shown in Fig.35.

The results of 3 experiments where calcium-evoked responses were obtained in the presence of either 10 or 20 mM TEA are presented in Tables 18A, 18B and 18C.

DISCUSSION

The ability of quaternary ammonium ions to block a variety of potassium channels, including calcium-activated ones, is well documented (see review by Lattore & Miller, 1983). It is therefore not surprising that these compounds were found to exert a similar blocking action on Ca^{2+}-activated K^+-channels of hamster eggs.

The difference in blocking potency between choline

Figure 35

The blocking action of tetraethylammonium (TEA) on the calcium-evoked hyperpolarization. Responses obtained in normal solution are presented in A. The responses illustrated in B and C were obtained in the presence of 20mM external TEA. Responses elicited after recovery from TEA treatment are shown in D.

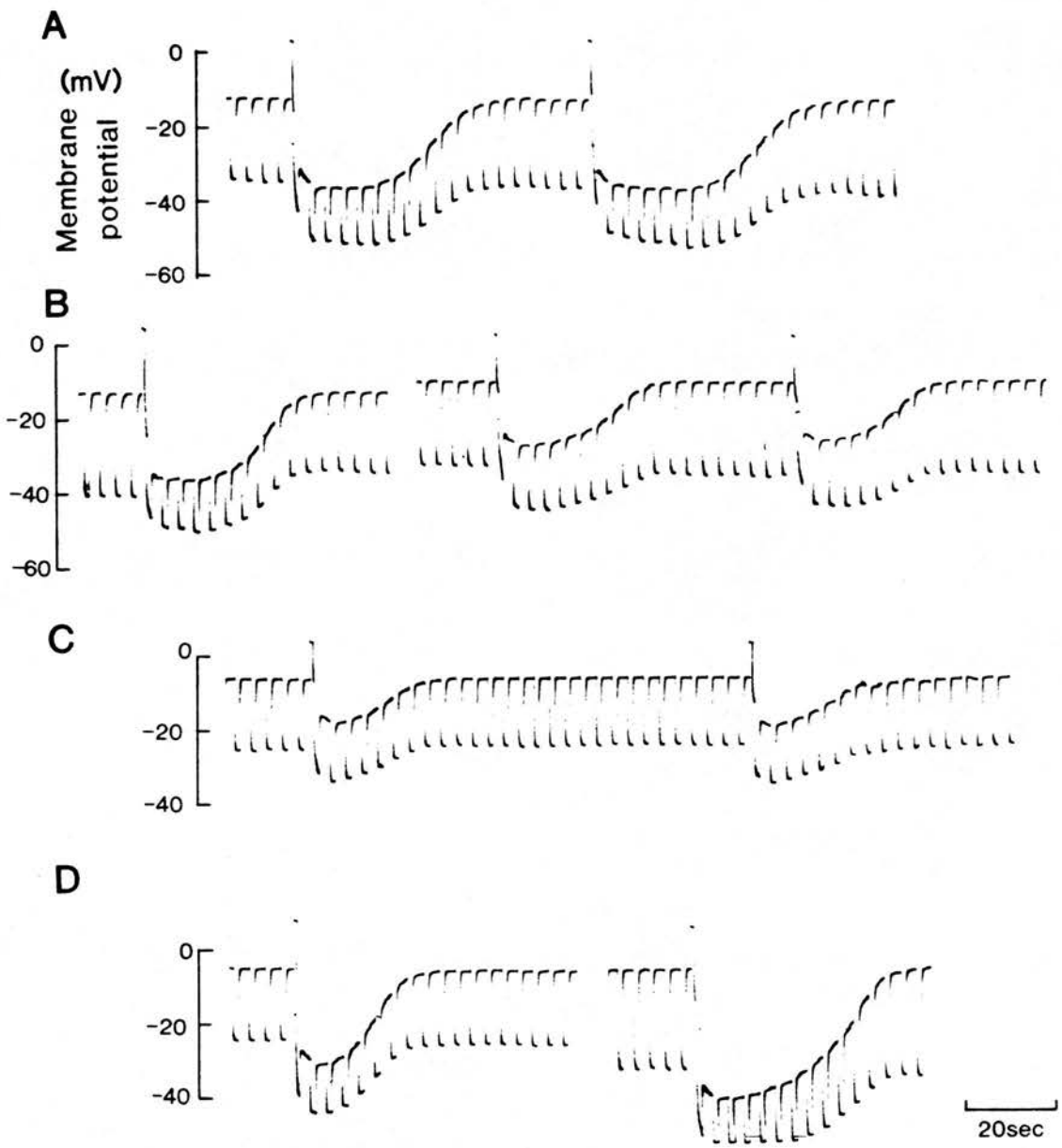

A
Membrane potential (mV)
0
-20
-40
-60
B
0
-20
-40
-60
C
0
-20
-40
D
0
-20
-40
20sec

Table 18[A]

The effect of TEA on various parameters that characterize the calcium-evoked hyperpolarization.

	CONTROL				
Experiment No.	E_r (mV)	$T_{\frac{1}{2}}$(V) (sec)	$\frac{Ro-Rt}{Ro}$ as %	V (mV)	ΔV (mV)
1	-	24	-	-16	6.0
	-	19	-	-17	10.0
	-	40	-	-16	9.0
	-	50	-	-15	14.0
	-	27	-	-15	11.0
2	-	14	-	-27	11
	-	27	-	-33	17
	-	30	-	-26	17
	-	28	-	-29	18
3	-61	26	50	-15	23
	-93	17	31.5	-14	25

Table 18B
The effect of TEA on various parameters that characterize the calcium evoked hyperpolarization.

Experiment No.	E_r (mV)	TEA $T_{\frac{1}{2}}$(V) (sec)	$\frac{Ro-Rt}{Ro}$ as %	V (mV)	ΔV (mV)
1	-	40	-	-14	10.0
	-	35	-	-13	5.6
	-	--	-	-16	0.0
	-	20	-	-14	4.0
	-	--	-	-13	1.0
2	-	15	-	-32	8
	-	11	-	-40	7
	-	10	-	-33	12
	-	9	-	-35	9
3	-78	26	40	-13	26
	-86.5	23	22	-10	17
	-70	21	25	-10	15
	-45	51	35	-8	13

Table 18 C

The effect of TEA on various parameters that characterize the calcium evoked hyperpolarization.

	CONTROL		RECOVERY		
Experiment No.	E_r (mV)	$T_{\frac{1}{2}}$(V) (sec)	$\frac{Ro-Rt}{Ro}$ as %	V (mV)	ΔV (mV)
1	-	30	-	-13	3.5
	-	-	-	-14	6.0
	-	26	-	-15	6.0
	-	29	-	-16	6.0
2	-	7	-	-37	6
	-	11	-	-33	11
	-	12	-	-33	9
	-	9	-	-34	9
3	-85	18	30	-8	23
	-82	63	41	-4	32
	-69	33	54	-3	36

Control solution in experiment 3 refers to the sodium-free lithium-containing solution.
The concentration of TEA was 10mM for experiments 1 and 2 and 20mM for the remainder.

and TEA might be related to their molecular size. The molecular cross-sectional area of choline is approximately 23A whereas that of TEA is approximately 37A (Coranado & Miller, 1982). It is therefore feasible that Ca^{2+}-activated K^{+}-channels of hamster eggs are weakly permeant to choline and probably completely impermeant to TEA. Coronado and Miller (1982) reported that the channel conductance of a reconstituted potassium selective channel derived from the sarcoplasmic reticulum of rabbit muscle, drops abruptly when the molecular cross-section of the traversing cation exceeds 18-20A. Perhaps a similar steric constraint operates in Ca^{2+}-activated K^{+}-channels of hamster eggs.

The steady membrane hyperpolarization observed upon substitution of sodium with choline, has also been observed in mouse eggs (Powers & Tupper , 1974; Okamoto, Takahasti & Yamashita, 1977). Powers and Tupper and Okamoto et. al. attributed this to the presence of a substantial resting sodium conductance which is reduced when a relatively large cation, like choline, is used to replace external sodium. A similar explanation might apply in hamster eggs. Alternatively the choline induced steady hyperpolarization might reflect a reduced leak conductance between the membrane and the tip of the microelectrode.

Igusa and Miyazaki (1983) reported that the duration of the calcium evoked response is considarbly

extended when lithium is used as a substitute for external sodium. To explain this they suggested the presence of a Na^{+}-Ca^{2+} exchange, which is known to be suppressed by external lithium (see review by Baker, 1972). The results of this section seem to be at variance with the results of Igusa and Miyazaki (1983) since response prolongation was not observed in the presence of lithium. Since their experiments were conducted at 33°C whereas these experiments were carried out at 23°C, the temperature difference may account for the discrepancy if lithium inhibits the Na^{+}-Ca^{2+} exchange system to a lesser extent at the lower temperature than at the higher. Consequently enhancement of the duration of the response would be less evident at the lower temperature.

GENERAL DISCUSSION

Intracellular recordings from hamster eggs have revealed the presence of a leak conductance which is generated at the moment of microelectrode impalement. Therefore the recording of the true membrane potential in these eggs requires that the leak conductance is minimized (i.e., the seal resistance between the microelectrode and the egg membrane is of the order of several GΩ). Independent estimates of the membrane potential of hamster eggs based on data derived from tracer flux experiments have not yet been carried out and hence the degree of sealing during microelectrodes recordings cannot be assessed without the use of certain assumptions (see Discussion in Chapter II).

Experiments designed to investigate systematically the effect of K^+, Na^+ and Cl^- on the membrane potential of hamster eggs are needed in order to determine the permeability ratios of the different ions. Although the majority of egg membranes in different species have resting potentials primarily governed by the K^+ permeability (see review by Hagiwara & Jaffe, 1979) exceptions have been reported. For example Dascal, Landau & Lass (1984) have reported that the membrane of Xenopus eggs has an appreciable Na^+ permeability ($P_{Na^+}/P_{K^+} = 0.12$) which plays a major role in determining the resting membrane potential. A similar role for the Na^+ permeability in determining the resting potential of the egg of the freshwater fish Oryzias has

been reported by Nuccitelli (see review by Hagiwara & Jaffe, 1979). Intracellular recordings from the eggs of the marine worm Urechis caupo have shown that the membrane potential shifted by 20mV for a tenfold change in $[K^+]_o$ and by 10mV for a tenfold change in $[Na^+]_o$, which suggests that Na^+ contributes significantly in determining the resting potential of these eggs.

The observation that large changes in extracellular potassium have little effect on the membrane potential of hamster eggs has been reported by Miyazaki and Igusa (1982). The membrane potential of mouse eggs has also been shown to be relatively insensitive to large shifts in external potassium (Okamoto, Takahashi & Yamashita, 1977). These workers report that a 5-fold change in external potassium depolarized mouse eggs by about only 5mV, instead of the expected 40mV change predicted by the Nernst equation (provided, of course, that the egg membrane acts as an ideal potassium electrode). Part of this insensitivity towards changes in external potassium may be due to the presence of a leak around the microelectrode. On the other hand it may be that the membrane potential of unfertilized hamster and mouse eggs is dictated by ions other than potassium and in particular by sodium or chloride. The enhancement of a background potassium conductance might begin after egg activation has commenced and perhaps caused by an elevation of intracellular pH as observed in the sea urchin (see review by Whitaker & Steinhardt, 1982) Patch clamp

studies, have in fact revealed that changes in pH can affect both voltage dependent (Labarca,Coronado & Miller, 1980) and Ca^{2+}-activated K^{+}-channels (Cook, Ikeuchi & Fujimoto, 1984). In both these investigations a rise in intracellular pH promotes whereas a fall in pH inhibits channel opening. Chloride channels on the other hand exhibit quite the opposite behaviour towards alterations of intracellular pH (Hanke & Miller, 1983).

Igusa and Miyazaki (1983) have reported that following insemination, the membrane potential of mouse eggs became less responsive to changes in external chloride, than did the potential of unfertilized eggs. This suggests an increased membrane permeability to another ion. Furthermore, these workers observed that injection of EGTA had no significant effect on the observed steady hyperpolarizing shift of the membrane potential following sperm egg fusion. They therefore proposed that it is mainly due to the development of a potassium conductance that is calcium-insensitive. If this is the case then fertilized eggs should respond to changes in external potassium in a more Nernstian fashion than unfertilized eggs do.

It has already been mentioned that in the hamster sperm-egg fusion initiates a series of hyperpolarizing responses produced by periodic opening of Ca^{2+}-activated K^{+}-channels (see Introduction in Part II of chapter II). Igusa & Miyazaki (1983) have shown that these sperm-evoked hyperpolarizations are critically

dependent on external calcium. In addition they also observed that the frequency of occurrence of these sperm-evoked responses is drastically decreased in the presence of the calcium channel blockers cobalt (Co^{2+}) and manganese (Mn^{2+}) or by injecting the eggs with the calcium chelating agent EGTA. Igusa & Miyazaki therefore suggested that the recurring hyperpolarizations might be caused by continuous calcium influx across the egg membrane. It is difficult to see how such an influx can arise since the voltage-sensitive calcium channels found in hamster eggs are completely inactivated in the voltage range examined by Igusa and Miyazaki. The absence of a pronounced depolarizing phase prior to the onset of a sperm-evoked hyperpolarization also argues against the hypothesis that a calcium influx across the cell membrane is responsible for the observed activation of potassium channels. Furthermore, if indeed persistent calcium influx operates across the oolemma, the question arises as to why is it that periodic activation of Ca^{2+}-activated K^{+}-channels is not observed in unfertilized hamster eggs. One possibility might be that calcium selective channels distinct from the voltage-gated ones that are already present in eggs, are added to the egg membrane during gamete fusion. Alternatively sperm function may not be confined to transfer of genetic material only but it may be a more active one which involves cycles of calcium release and re-uptake by the sperm itself (much like the

sarcoplasmic reticulum of skeletal muscle).

The marked prolongation of the calcium-evoked response in the presence of external lanthanum probably suggests the presence in hamster eggs of a surface located calcium pump. The enhancement of the duration of the response observed in the presence of high concentrations of external calcium is probably caused by partial inhibition in the activity of the calcium pump. If the rate at which the "calcium pump" extrudes calcium is increased by membrane hyperpolarization then a possible role for the periodic activation of Ca^{2+}-activated K^{+}-channels observed during fertilization might be to assist in removing calcium by enhancing the activity of the pump.

The importance of the periodic activation of the Ca^{2+}-activated K^{+}-channels during fertilization can probably be assessed by blocking them and determining what part of the fertilization process is affected. TEA, barium and choline could be used for investigations of this sort and in addition they could also be usefully employed in determining the possible role of Ca^{2+}-activated K^{+}-channels during the repolarizing phase of the calcium-dependent anode-break response. This predominantly calcium action potential may acquire greater significance once egg activation has commenced and perhaps if unfertilized mammalian eggs are indeed excitable, it may play a role during the initial sperm-egg interaction. Although at present, the experimental evidence does not support

such a role for the calcium spike, improvement in the recording techniques (eg whole cell giga-seal recordings using patch electrodes) might help shed more light as to the true value of the membrane potential and the possible functional significance of voltage-gated calcium channels in mammalian eggs.

APPENDIX 1

The Wong, Lecar and Adler model assumes that channel opening is effected by the simultaneous binding of N calcium ions (Ca^{2+}) to an allosteric binding site of the channel protein. They propose that the binding constant K is

(a) $\propto [Ca^{2+}]^N$ and

(b) $\propto \exp(AV)$

where $A = nF/RT$

Hence $K = K'[Ca^{2+}]^N \exp(AV)$.....(1)

Let the transition from the closed to the open state be governed by the microscopic rate constant k_o and the reverse transition by k_c.

From equation (1)

$$\ln K = N \ln K'[Ca^{2+}] + AV$$

When $V = V_o$ (where V_o is the voltage at which 50% of all the channels are open) then $K = k_o/k_c = 1$ and

$$N \ln K'[Ca^{2+}] = AV_o$$

Hence $$V_o = -\frac{N \ln K'[Ca^{2+}]}{A}$$

A can be obtained by differentiating equation (1) as follows

Let $u = V = V-V_o$

Using the chain rule

$$\frac{dp}{dV} = \left(\frac{dp}{du}\right)\left(\frac{du}{dV}\right)$$

Now $$\frac{dp}{du} = \frac{d}{du}[1+\exp(-Au)]$$

$$= -1[1+\exp(-Au)]^{-2}$$

Now $$\frac{dp}{dV} = \frac{d}{dV} -A(V) = -A$$

Therefore $$\frac{dp}{dV} = \frac{A}{1 + \exp[A(V-V_o)]^2}$$

At $V = V_o$

$$\frac{dp}{dV} = \frac{A}{4} \quad \text{and hence } A = 4\ (dp/dV)$$

From equation (1) a tenfold change in the free calcium concentration produces a shift of 2.3(N/A)mV. Knowing A, N can be calculated. Wong, Lecar and Adler found that the value of N that gave the best fit was 3, that is 3 calcium ions had to bind simultaneously to receptor sites on the channel protein in order for the channel to open.

APPENDIX 2

Consider an equivalent circuit consisting of two parallel conductances, g and g_K, each in series with a battery V and E_K respectively. Furthermore at rest g_K is an open circuit, which can be connected into the rest of the circuit via a switch. When the switch is closed the value of the membrane potential V' is given by:

$$V' = \frac{g_K E_K + gV}{g + g_K} \quad \text{.....(1)}$$

Hence the difference in voltage prior to and following closure of the switch is:

$$V = V'-V = \left(\frac{g_K E_K+gV}{g+g_K}\right) - V \quad \text{.....(2)}$$

The above equation can be rearranged to:

$$V = \frac{g_K}{gK+g} \quad (EK-V) \quad \text{.....(3)}$$

Solving for E_K in the equation above we get:

$$EK = \left(\frac{g_K+g}{g_K}\right)\Delta V + V \quad \text{.....(4)}$$

Provided that the total current flow during an electrotonic potential prior to closing the switch is equal to the total current flow after closing the switch, it follows that:

$i = (g_K+g)\ p = Pg = \text{constant}$ ...(5) , where

P is the amplitude of the electrotonic response prior to closing the switch and p is the amplitude of the

electrotonic response after closing the switch.

Hence $$\frac{g_K+g}{g} = \frac{P}{p} \quad \text{.....(6)}$$

Rearranging and solving for g_K in the equation above we get:

$$g_K = \left(\frac{P-p}{p}\right) g \quad \text{.....(7)}$$

Substituting for g_K in equation (6) using the result of equation (7) we get that

$$\frac{g_K+g}{g_K} = \frac{g + \left(\frac{P-p}{p}\right)}{\left(\frac{P-p}{p}\right) g} \quad \text{.....(8)}$$

$$= \frac{P}{P-p}$$

Substituting $[(g_K+g)/g_K]$ for $[(P-p)/P]$ in equation (4) we obtained that

$$E_K = E_r = \left(\frac{P}{P-p}\right)\Delta V + V$$

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CALCIUM ACTION POTENTIALS IN UNFERTILIZED EGGS OF MICE AND HAMSTERS

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SUMMARY

Measurements of membrane potential and resistance have been made in zona-free eggs of mice and hamsters. The mean ± S.D. values for membrane potential were −91 ± 28 mV (mouse) and −97 ± 29 mV (hamster) and for input resistance were 430 ± 230 MΩ (mouse) and 410 ± 150 MΩ (hamster) respectively. Large fluctuations (20 mV) of membrane potential occurred apparently at random and these were accompanied by changes of membrane resistance. Depolarizing current pulses passed through the recording micro-electrode evoked action potentials in eggs of both species. The threshold for excitation was about −50 mV, the maximum rate of rise of the action potential was about 3 $V.s^{-1}$ and its peak value was about +13 mV. Action potentials could be evoked in eggs bathed in sodium-free solution or in normal solution containing tetrodotoxin (3 μM). The presence of cobalt (5–20 mM), lanthanum (1 mM) or verapamil (200–400 μM) in the bathing solution suppressed the action potential. Raising the extracellular calcium concentration from 4 to 40 mM increased the peak value of the action potential by 25 mV. It is concluded that the plasma membranes of mouse and hamster eggs have voltage-dependent calcium channels.

INTRODUCTION

Action potentials have been recorded from the eggs of starfish (Miyazaki, Ohmori & Sasaki, 1975) and sea urchins (Jaffe, 1976; Okamoto, Takahashi & Yamashita, 1977). These regenerative responses arise from the opening of voltage-dependent calcium channels. The possible role of action potentials in the fertilization of invertebrate eggs has been discussed by Hagiwara & Jaffe (1979) and Whitaker & Steinhardt (1982). By comparison, electrophysiological studies of mammalian eggs are not so extensive. It is known, however, that the eggs of hamsters and mice give anode-break responses to hyperpolarizing current pulses (Okamoto *et al.* 1977; Miyazaki & Igusa, 1982). The characteristics of the anode-break responses suggest that they are caused by the opening of calcium channels apparently inactivated in the resting state. Evidence from voltage-clamp experiments on mouse eggs (Okamoto *et al.* 1977) supports the idea that there are voltage-sensitive calcium channels which are inactivated at the resting potential usually observed in these eggs. Recently, however, it has been shown by Georgiou, Bountra, Bland & House (1983) that the membrane potential and input resistance of hamster eggs are substantially larger than previously recorded. It is probable that the earlier measurements of potential and resistance in mammalian eggs suffered from impalement leak artifacts as was the case for intracellular recordings from sea urchin eggs (Hagiwara & Jaffe, 1979; Whitaker & Steinhardt, 1982).

In this paper we report that mouse eggs, like hamster eggs, have large membrane potentials and input resistances and that these mammalian eggs have calcium action potentials which can be evoked by depolarization.

A preliminary account of some aspects of this work has been given to the Physiological Society (Bland, Bountra, Georgiou & House, 1983).

METHODS

Egg donors

Mature female mice (Balb/c strain) and golden hamsters were egg donors in this study.

Mice. Animals were maintained under a fixed light/dark cycle (16 h light/8 h dark). Super-ovulation was induced by injecting (I.P.) 10 i.u. pregnant mare serum gonadotrophin (PMSG) (Folligon, Intervet Labs. Ltd, Cambridge) in the early evening followed by injection (I.P.) of 10 i.u. HCG (Chorionic Gonadotrophin, CG-2 Sigma Chemical Co., St Louis, U.S.A.) forty-eight hours later. Animals were killed 15–18 h after the second injection and their oviducts were placed in a dish containing a physiological solution (called normal) at room temperature (20–22 °C). The compositions of the normal and test solutions are given in Table 1; solutions contained HEPES as a buffer. In some experiments a bicarbonate–CO_2-buffered solution (Table 1) was used and no difference in the electrical behaviour of mouse and hamster eggs was found. Eggs were removed from the oviducts and placed in normal solution containing hyaluronidase (1 mg.ml^{-1}, Type 1-S, Sigma) for 5 min to remove the cumulus oophorus. Removal of the zona pellucida was achieved by transferring eggs (freed from cumulus) into a normal solution containing protease (1 mg.ml^{-1}, Type XIV, Sigma) for 10–15 min at room temperature.

Hamsters. Details of obtaining zona-free eggs from hamsters were similar to those described for mice, with the following exceptions. Animals were injected (I.P.) with 30 i.u. PMSG in the early evening and with 45 i.u. HCG 48 h later. About 16 h after the HCG injection animals were killed, the oviducts opened and eggs transferred into normal solution. To remove the cumulus each egg was incubated for 2–4 min in normal solution containing hyaluronidase (1 mg.ml^{-1}). To remove the zona pellucida each egg (freed from cumulus) was bathed for 1–3 min in normal solution containing trypsin (1 mg.ml^{-1}, Type III, Sigma). Enzyme treatments were carried out at room temperature (20–22 °C).

Intracellular recording

An egg was transferred to a chamber (volume 5 ml) mounted on an inverted microscope (Biovert, Reichert, Austria). Solution was pumped through the chamber by a Watson-Marlow HR flow inducer (MRHE 200) at a rate of about 5 ml.min^{-1}. The chamber contained a solid silver/silver chloride electrode in contact with the bathing solution. In experiments where the chloride concentration of the bathing solution was changed the bath was earthed via a 3 M-KCl saline–agar bridge.

A micro-electrode (30–80 MΩ) filled with 2 M potassium acetate was inserted into the egg for simultaneous potential recording and current passage. It was connected to the input of a high-impedance pre-amplifier (Model KS700, WP Instruments, U.S.A.). Current pulses from a Devices stimulator triggered by a Digitimer (D4030, Devices Ltd) were passed via a bridge circuit between the barrel of the micro-electrode and the bath electrode. Cell impalement was achieved by resting the micro-elecrode on the surface of the egg and increasing the negative capacity applied to the micro-electrode so that it went briefly into electrical oscillation.

RESULTS

All of the experiments described here were made on zona-free eggs of mice and hamsters. In separate experiments on eggs with intact zona pellucida similar results were found so the results presented below are not artifacts caused by the enzymic removal of the zona.

When a micro-electrode was inserted into a mouse egg the recorded membrane potential was initially small (−10 to −30 mV) and then began to increase with time. In about half of the cells examined the potential attained values of about −35 mV and the input resistance reached about 150 MΩ. For reasons which will be discussed later we consider that these cells were damaged by micro-electrode penetration and therefore they have been excluded from the present results. In the remainder of cells examined both the potential and the resistance increased together over a period ranging from 3 to 30 min to reach maximal values around −90 mV and 500 MΩ respectively. Similar progressive increases in potential and

Table 1. *Composition of solutions*

Solution	Na	K	Ca	Mg	Choline	Cl	Lactate	Pyruvate	Glucose	Sucrose
Normal	143·6	5	4	1·2	—	135·4	20	1·1	5	—
Sodium free	—	5	4	1·2	120	132·9	—	—	5	40
High calcium sodium free	—	5	40	1·2	40	124·9	—	—	5	90
High magnesium sodium free	—	5	4	40	40	130·5	—	—	5	80
Low chloride†	143·6	5	4	1·2	—	15·4	20	1·1	5	—
CO_2–bicarbonate*	146·1	5	4	1·2	—	115·4	20	1·1	5	—

Concentrations expressed as mM. All solutions except CO_2–bicarbonate solution contained 5 mM HEPES plus 2·5 mM-NaOH (or KOH) to give pH 7·2.

* The pH of the CO_2–bicarbonate solution was 7·4. This solution contained $NaHCO_3$ at a concentration of 25 mM.

† In most experiments sodium chloride was replaced by sodium methylsulphate; in others chloride was replaced by gluconate or propionate.

resistance after impalement have been observed in the eggs of starfish (Miyazaki *et al.* 1975), sea urchins (Jaffe & Robinson, 1978; Chambers & de Armendi, 1979) and the hamster (Georgiou *et al.* 1983). These authors have attributed the increases in potential and resistance to an improvement of the sealing of the micro-electrode to the cell membrane.

Fig. 1 *A* displays an illustrative example of an intracellular recording from a mouse egg. The pen trace (top) shows the increase in membrane potential with time. Shortly after impalement hyperpolarizing current pulses were passed through the micro-electrode for the period indicated by the horizontal bar. Electrotonic potentials appeared as downward deflexions and progressively increased in size. Over a range of membrane potential each was followed by an anode-break response which gave an upward deflexion. Eventually a membrane potential was reached at which anode-break responses failed to appear and thereafter the membrane potential and the electrotonic potential continued to increase. After about 3 min the current pulses were switched off and the membrane potential continued to climb to about −120 mV and showed spontaneous fluctuations of about 20 mV in amplitude. Brief depolarizing current pulses produced action potentials (●) and oscilloscope pictures of these responses are shown in Fig. 1 *Ba* below the pen trace. Occasionally spontaneous action potentials (marked ∗, Fig. 1 *A*) were recorded. It is possible that the progressive increase in potential and resistance observed within 5 min of impalement is caused by enhanced sealing of the micro-electrode to the cell. To test this idea a second micro-electrode was inserted into the egg during the passage of hyperpolarizing current pulses (horizontal bar) through the first electrode. Just before the second impalement the input resistance of the cell was 520 MΩ, indicated by the size of the electrotonic potential (lower oscilloscope trace, Fig. 1 *Bb*). Upon the second impalement the potential and the resistance fell transiently; the upper oscilloscope trace in Fig. 1 *Bb* shows the smaller electrotonic potential (accompanied by an anode-break response) shortly after the second impalement. Thereafter the membrane potential and the electrotonic potential recovered their high values and the spontaneous fluctuations of potential reappeared (Fig. 1 *Bc*).

The pen trace and oscilloscope pictures in Fig. 1 show several new features about the electrical behaviour of unfertilized mouse eggs, namely (a) high membrane potential and resistance, (b) large fluctuations of the potential 'at rest', (c) action potentials evoked by depolarization, and (d) spontaneous action potentials. These features, which we have recorded also in unfertilized hamster eggs, will now be discussed in detail.

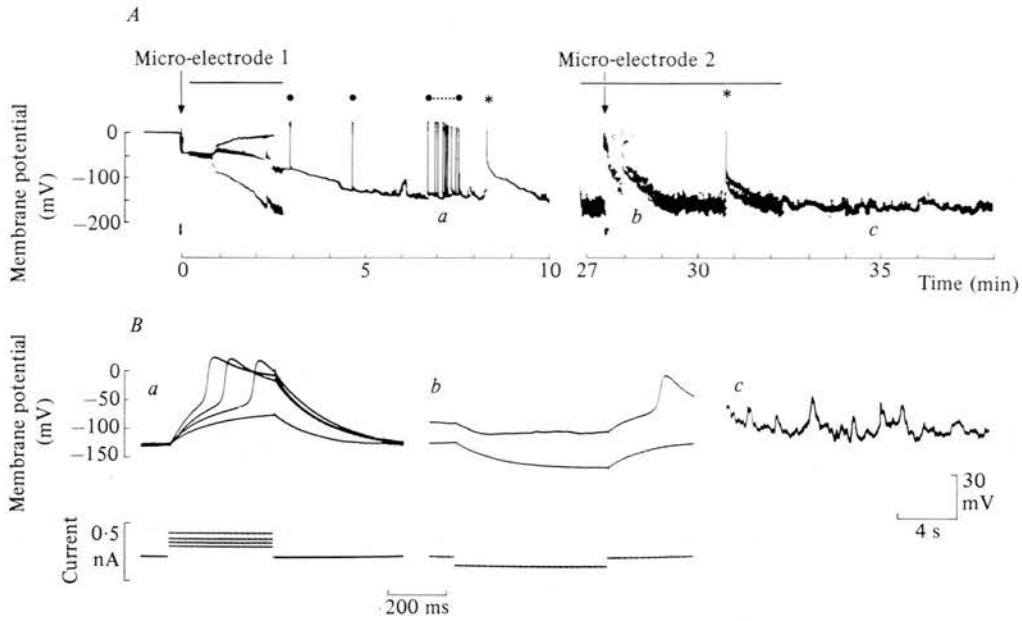

Fig. 1. An intracellular recording from a mouse egg. *A*, pen trace of membrane potential recorded by a micro-electrode (1) after its insertion. Horizontal bars indicate periods when hyperpolarizing current pulses (500 ms) were passed through micro-electrode 1. Brief depolarizing current pulses (●) evoked action potentials. Occasionally spontaneous action potentials (*) were recorded. A second micro-electrode (2) was inserted into the egg about 27 min after the insertion of micro-electrode 1. *B*, oscilloscope pictures of action potentials (*a*), electrotonic potentials (*b*) and spontaneous fluctuations of potential (*c*) recorded at various times after impalement. The times are indicated by *a*, *b* and *c* on the pen trace in *A* above.

Passive electrical properties

Membrane potential, resistance and capacitance

The current/voltage relations of twenty-two mouse eggs were obtained after allowing an appropriate period for the potential and resistance to attain their maximal values. Because the membrane potential fluctuates we estimated the average value from pen recordings for each cell. Table 2 shows the average membrane potential, resistance, time constant and capacitance for each egg. The mean values ($\pm$S.D.) of the membrane potential, resistance and capacitance were -91 ± 28 mV, 430 ± 230 MΩ and 280 ± 110 pF. Hamster eggs also had large membrane potentials and resistances (Table 2). In separate experiments on mouse eggs we measured the diameters of fifteen eggs and found a mean of 80 μm which is close to that reported for hamster eggs (Georgiou *et al.* 1983). On the assumption that the egg's surface equals the apparent area the specific membrane resistance and capacitance of mouse eggs were found to be 86 kΩ . cm^2 and 1·4 μF . cm^{-2} respectively. The values for hamster eggs were 82 kΩ . cm^2 and 3·3 μF . cm^{-2}. If the difference between the membrane capacitances of hamster and mouse eggs reflects a difference in their membrane areas (in spite of their similar diameters) then the above value of the membrane resistance of hamster eggs is an underestimate.

Effect of ionic substitution

When the sodium in the normal solution was replaced by choline the membrane potential increased. This suggests that the relative sodium permeability of the mouse egg is not

Table 2. *Electrical properties of eggs*

Egg	Average membrane potential (mV)	Resistance (MΩ)	Time constant (ms)	Capacitance (pF)
		Mouse eggs		
1	−106	240	100	420
2	−88	470	110	230
3	−63	530	120	220
4	−115	500	92	180
5	−130	660	130	200
6	−109	450	110	240
7	−56	800	170	210
8	−77	520	160	310
9	−100	1100	250	230
10	−54	250	85	340
11	−98	300	65	220
12	−104	310	39	130
13	−130	520	130	250
14	−60	570	65	110
15	−88	450	130	290
16	−73	270	59	220
17	−95	330	150	460
18	−165	400	110	270
19	−75	130	38	290
20	−78	260	69	270
21	−76	240	92	390
22	−59	110	69	600
Mean ± S.D.	−91 ± 28	430 ± 230	110 ± 50	280 ± 110
		Hamster eggs		
1	−95	430	210	490
2	−150	520	220	420
3	−71	620	390	630
4	−85	350	350	1000
5	−75	340	250	740
6	−107	180	120	670
Mean ± S.D.	−97 ± 29	410 ± 150	260 ± 100	660 ± 200

negligible. In ten experiments the mean (±S.D.) hyperpolarization produced by bathing mouse eggs in sodium-free solution was 34 ± 24 mV. A similar hyperpolarization occurred in hamster eggs.

Replacing most of the chloride in the bathing solution by an impermeant anion (see Methods) led to a mean (±S.D.) depolarization of 19 ± 6·6 mV (six mouse eggs); in two hamster eggs chloride replacement led to a depolarization of about 5 mV. Membrane resistance increased in all eggs bathed in low chloride solution.

Previous electrophysiological studies (Powers & Tupper, 1974; Okamoto *et al.* 1977; Miyazaki & Igusa, 1982; Georgiou *et al.* 1983) have reported a slight influence of potassium ions on the resting potential of mouse and hamster eggs. However, these experiments were carried out on eggs depolarized by impalement-leak artifacts and therefore it was necessary to re-examine the effect of potassium on potential and resistance. Raising the potassium concentration from 5 to 40 mM by addition of potassium chloride to a sodium-free solution caused a mean (±S.D.) depolarization of 35 ± 26 mV (six mouse eggs) somewhat smaller than the 52 mV expected from the Nernst equation. In one experiment replacement

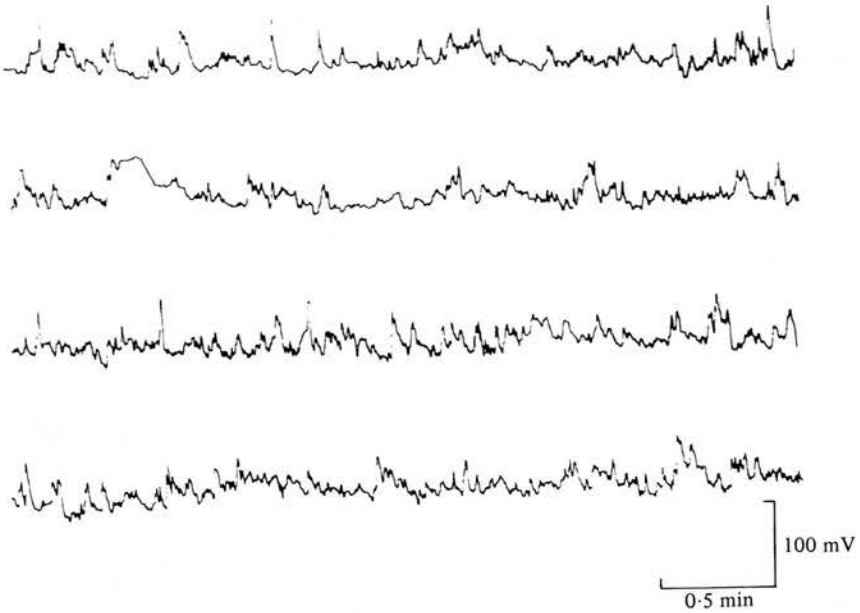

Fig. 2. An intracellular recording from a mouse egg showing spontaneous fluctuations of the membrane potential. The pen traces comprise a continuous excerpt from a longer period of recording. The average membrane potential of this egg was −160 mV and its input resistance was about 1000 MΩ.

of sodium in normal solution by equimolar amounts of potassium to give a final concentration of potassium equal to 25 mM caused a depolarization of 20 mV. Depolarization in all eggs examined was accompanied by a fall in resistance. In all of the above experiments the cells retained their excitability in the solutions with raised potassium concentrations.

An increase of the calcium concentration from its normal value of 4 mM to 40 mM was made in sodium-free solution (Methods). This change usually caused an irreversible depolarization. The input resistance fell during the prolonged depolarizing response to elevated calcium concentration. These results are at variance with previous findings in mammalian eggs (Okamoto *et al.* 1977; Miyazaki & Igusa, 1982). It is possible that the hyperpolarization and increase in resistance observed previously arose from a reduction of the shunt at the tip of the recording micro-electrode.

Active membrane properties

Spontaneous potential fluctuations

Fig. 2 shows a set of continuous intracellular recordings from a mouse egg which exhibits spontaneous fluctuations of membrane potential. Inspection of these traces indicates that the fluctuations are due to waves of depolarization which appear to occur randomly. No detailed analysis of these records has been made. It was observed that the potential fluctuations were accompanied by changes in membrane resistance. In two typical records the resistance fell at the peaks of the depolarizations to about half of its value at the feet of the depolarizing waves. The potential fluctuations were not an invariable feature of eggs with high membrane potentials since they could be selectively abolished by addition of 1 mM lanthanum to the bathing solution. This suggests that they might arise from the random opening of calcium channels since lanthanum ions block voltage-dependent calcium channels (Hagiwara & Byerly, 1981).

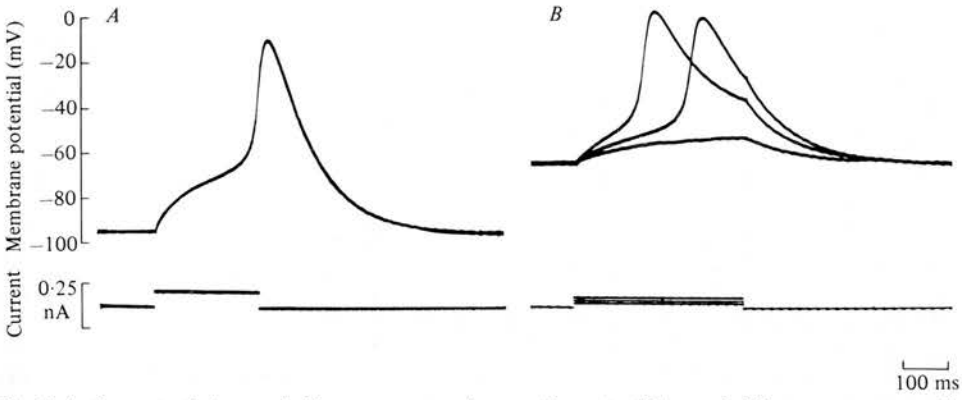

Fig. 3. Action potentials recorded in mouse eggs. *A*, an action potential recorded from a mouse egg stimulated by a depolarizing current pulse (lower trace) passed through the recording micro-electrode. *B*, action potentials in a different egg evoked by depolarizing current pulses of different amplitudes. A subthreshold electrotonic potential is shown also.

Table 3. *Characteristics of action potential in mouse eggs bathed in sodium-free solution*

Egg	Membrane potential (mV)	Threshold (mV)	Action potential peak (mV)	Amplitude of action potential (mV)	Maximum rate of rise ($V.s^{-1}$)
1	−151	−37	+25	176	4·9
2	−131	−57	−6	125	2·1
3	−129	−40	+16	145	2·4
4	−107	−49	+25	132	3·4
5	−132	−57	+4	136	2·2
Mean ± S.D.	−130 ± 16	−48 ± 9	+13 ± 14	143 ± 20	3·0 ± 1·2

Action potentials

A brief depolarizing current pulse passed through the recording micro-electrode evoked an action potential (Fig. 3*A*). In most eggs the action potential was initiated when the membrane potential reached about −50 mV and it attained a positive value at its peak. Measurements were made on action potentials recorded from five mouse eggs to get precise information about threshold, peak value and the maximum rate of rise. In each egg at least four action potentials were analysed and the mean values for the entire study are given in Table 3.

When the amplitude of a depolarizing current pulse sufficient to evoke an action potential was increased the action potential occurred earlier and the peak was increased somewhat (Fig. 3*B*). The increase in the peak value with increase in current strength allowed an estimate of the 'active resistance' to be obtained (cf. Fatt & Ginsborg, 1958). Generally the 'active resistance' at the peak of the action potential was about 100 MΩ as opposed to the 'resting resistance' of about 500 MΩ. This fall in resistance during the action potential indicates the presence of ion channels opened by membrane depolarization.

It was not possible to evoke action potentials at a rate exceeding about 1 Hz. This suggests that the refractory period is about 1 s. Fig. 4 shows a train of action potentials elicited by

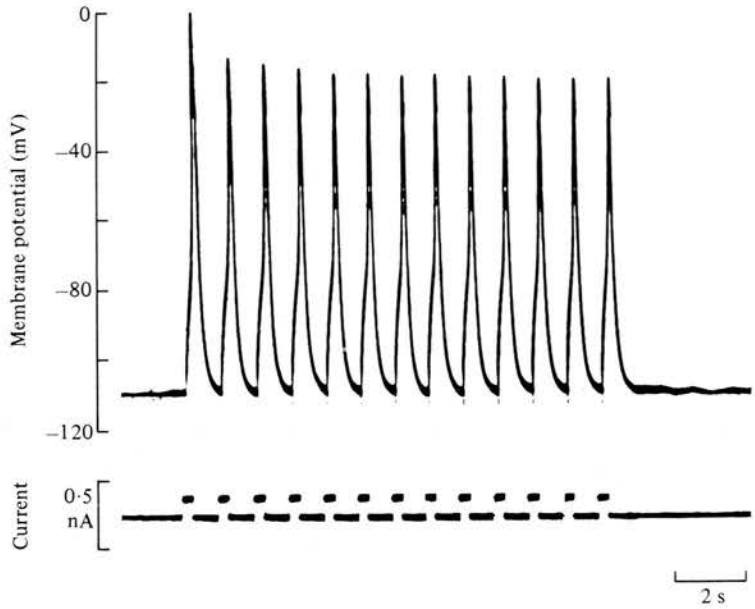

Fig. 4. Successive action potentials (upper trace) evoked by a train of depolarizing current pulses (lower trace) applied to a mouse egg.

a series of depolarizing current pulses. The peak value of successive active potentials declined until a new constant level was reached. Perhaps this decline indicates the presence of a type of channel inactivation similar to that suggested for the inactivation of calcium channels by calcium entry in molluscan neurones (Tillotson, 1979). Another possibility is that during a series of action potentials there is a progressive accumulation of the ion carrying the inward current at the inner surface of the membrane. An explanation of this kind was put forward by Niedergerke & Orkand (1966) to account for the progressive decrease in the overshoot of frog ventricular action potentials, the accumulation of calcium ions causing a fall in the equilibrium potential for calcium.

Ionic basis of action potential

Action potentials could be evoked in mouse and hamster eggs bathed in sodium-free solution (Fig. 5) thus excluding the possibility that the action potential arises from sodium influx. Moreover, the action potential was not reduced in the presence of high concentrations (3 μM) of tetrodotoxin, a sodium channel blocker.

To test whether the action potential is due to the opening of calcium channels two kinds of experiments were performed. In the first the extracellular concentration of calcium was raised from 4 to 40 mM in sodium-free solution. In the second kind the effects of various calcium channel blockers were tested on the egg's action potential.

Fig. 6 shows that raising the calcium concentration in the bathing solution increased both the threshold for initiation of the action potential and the peak value of the action potential in a mouse egg. In similar experiments on ten eggs the mean ($\pm$S.D.) increase in the peak value was $25 \pm 3{\cdot}7$ mV which is not significantly different from the shift (29 mV) expected for a tenfold change in calcium concentration according to the Nernst equation.

It has been established that magnesium ions cannot substitute for calcium as charge carriers in voltage-sensitive calcium channels (Hagiwara & Byerly, 1981). When calcium was

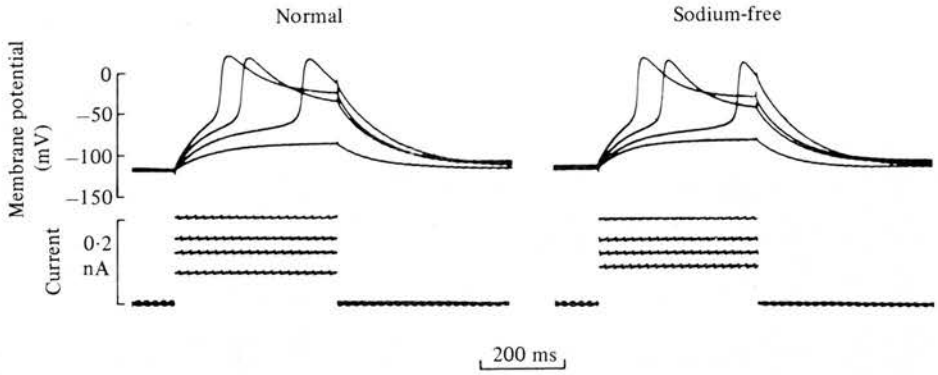

Fig. 5. Action potentials recorded from a mouse egg bathed in normal solution (left) and then in sodium-free solution (right). The records (right) were obtained 7 min after change to sodium-free solution and during the long tail of a spontaneous depolarization; subsequently the resting potential attained −145 mV but peaks of evoked action potentials were similar to those illustrated.

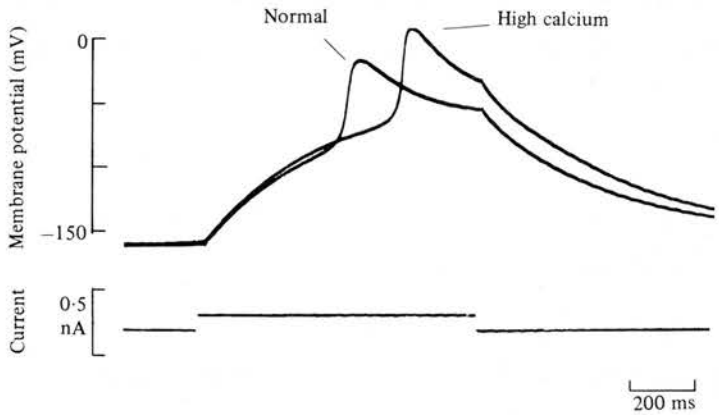

Fig. 6. Effect of raising the extracellular concentration of calcium on the peak value of the action potential recorded from a mouse egg. The calcium concentration was raised from the normal value of 4 mM to 40 mM in a sodium-free solution bathing the egg and the corresponding action potentials have been superimposed.

replaced by magnesium in the solution bathing mouse eggs the size of action potentials was severely reduced. Moreover, the threshold for excitation was raised. Frequently the size of the action potential did not recover fully upon superfusing the eggs with normal solution. However, full-sized action potentials were observed when external calcium concentration was raised to 40 mM.

Calcium channel blockers

Extensive electrophysiological evidence supports the idea that calcium channels can be blocked by a variety of cations including cobalt, manganese and lanthanum and also organic compounds such as verapamil (Hagiwara & Byerly, 1981; Reuter, 1983). It was of interest, therefore, to examine the effects of some calcium channel blockers on the egg's action potential.

Inorganic cations. Cobalt suppressed the action potential when it was present in the

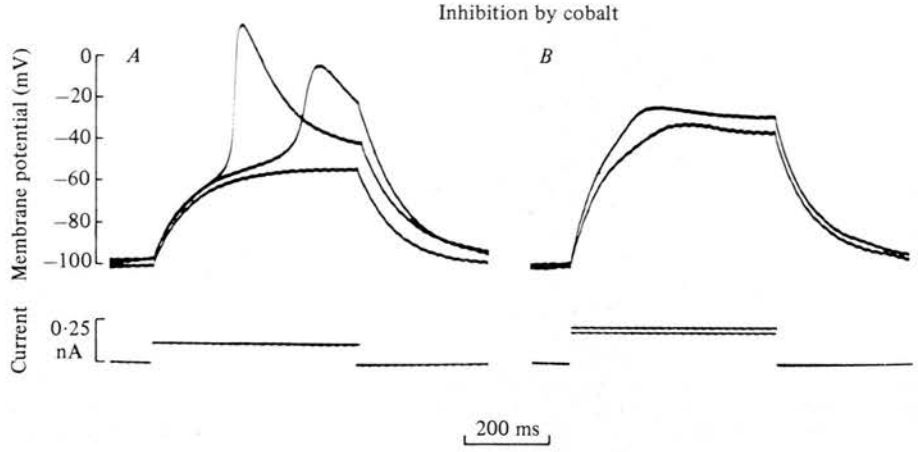

Fig. 7. Effect of cobalt on the action potential recorded from a mouse egg. Superimposed traces of responses to depolarizing current pulses recorded from the egg bathed in normal solution containing 5 mM cobalt chloride. *A*, records show progressive decline of action potentials recorded at 1, 3 and 5 min after solution containing cobalt had entered the chamber. *B*, depolarizations evoked by current pulses of increased amplitude applied to the same egg bathed in normal solution containing 20 mM cobalt chloride. Note that small residual active response persists in the presence of high concentration of cobalt.

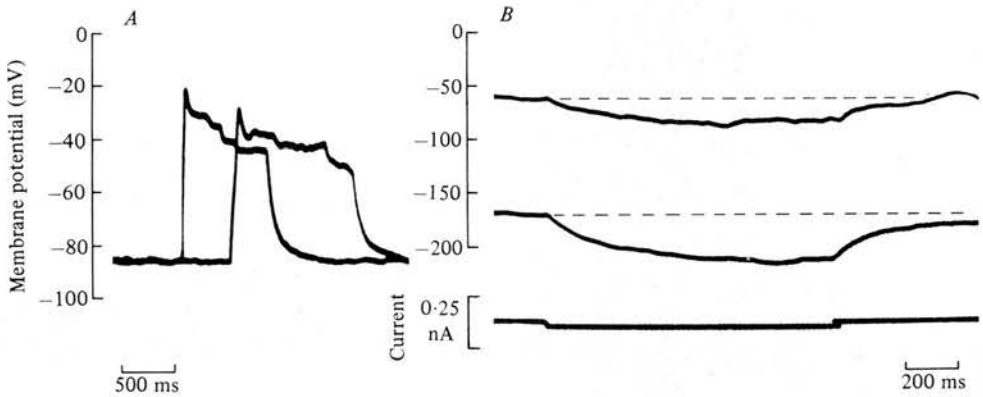

Fig. 8. Spontaneous action potentials recorded from mouse eggs bathed in normal solution. *A*, superimposed spontaneous action potentials in an egg. *B*, electrotonic potentials (upper traces) caused by identical hyperpolarizing current pulses (lower trace) applied to another egg during the plateau of a spontaneous action potential and after its end.

bathing solution at a concentration in the range 5–20 mM (Fig. 7). It is possible that the inhibition of the action potential illustrated in Fig. 7*A* was produced because cobalt raised the threshold for excitation. To examine this possibility the effect of increasing the depolarizing current pulse was recorded. An action potential was not evoked although a small non-linear response remained (Fig. 7*B*). The inhibition caused by cobalt was reversible.

Lanthanum blocked the action potential completely when it was present in the sodium-free solution at 1 mM. This agent also caused a marked hyperpolarization. Recovery was poor unless the bathing solution contained a calcium concentration of 40 mM.

Verapamil. In both mouse and hamster eggs verapamil (10 μM) failed to block the action

potential even after repetitive stimulation. It did produce, however, a 'use-dependence' block at a concentration in the range 200–400 μM as was found at lower concentrations in a voltage-clamp study of the calcium inward current in cat papillary muscle (Ehara & Daufmann, 1978).

Spontaneous action potentials

Occasionally large spontaneous depolarizations were observed. These sometimes attained positive peak values and some had very prolonged plateau phases (Fig. 8*A*). These spontaneous events basically resembled the evoked action potentials and so have been called spontaneous action potentials. Their mean peak value was about -10 mV. It was possible, on a few occasions, to record electrotonic potentials (produced by current pulses through the recording micro-electrode) during and after the plateau phase of a spontaneous action potential. An example is illustrated in Fig. 8*B*. Evidently the membrane resistance is lower during the plateau phase which suggests that a prolonged rise in ionic permeability underlies this phase. The ionic basis of the plateau phase has not been examined because spontaneous action potentials are relatively infrequent and therefore not amenable to controlled studies.

DISCUSSION

The results of this study are at variance with the published reports of other workers in two important respects.

First, we have recorded large membrane potentials and resistances in hamster and mouse eggs. Table 4 shows the available data on the electrical properties on the unfertilized eggs of the mouse, hamster and rabbit. In a previous paper we found that the recorded membrane potential of zona-free hamster eggs lay in the range -9 to -100 mV and that the higher potentials were associated with larger input resistances (Georgiou *et al.* 1983). Evidence was presented to show that low potentials arose from substantial leak artifacts at the point of insertion of the micro-electrode. In the present paper evidence has been presented to demonstrate that impalement produces a drop in potential and resistance (Fig. 1). Provided sufficient time is allowed for sealing of the micro-electrode to the cell membrane the potential and resistance often climb back to their true values. Similar conclusions about impalement-leak artifacts have been drawn from intracellular recordings from eggs of starfish (Miyazaki *et al.* 1975) and sea urchins (Jaffe & Robinson, 1978; Chambers & de Armendi, 1979). Indeed Chambers & de Armendi (1979) found that the application of hyperpolarizing current apparently improved the sealing process at the micro-electrode tip as reported for *Aplysia* neurones (personal communication by E. Mayeri cited by Brown & Flaming, 1977). A possible explanation for the fact that we have obtained apparently better micro-eloctrode sealing than that achieved by other workers studying mammalian eggs is that we usually applied hyperpolarizing current pulses to the cell after impalement.

The second point of difference between our results and those of others on mammalian eggs is that we have recorded action potentials in response to brief depolarizing current pulses whereas they have found evidence for excitability only through anode-break responses. It is likely that the depolarized state of eggs in previous studies has inactivated the voltage-dependent channels. The characteristics of the channels opened by depolarization, namely their ability to pass calcium ions and their block by cobalt, lanthanum or verapamil, indicate that they are selective calcium channels (cf. Hagiwara & Byerly, 1981). The same conclusion has been reached on the basis of the analysis of anode-break responses in mouse and hamster eggs (Okamoto *et al.* 1977; Miyazaki & Igusa, 1982; Eusebi, Colonna &

Table 4. *Electrical measurements on mammalian eggs*

Membrane potential (mV)	Input* resistance (MΩ)	Excitable characteristics	Reference
		Mouse eggs	
−8 to +2	—	—	Cross, Cross & Brinster (1973)
−14	18	—	Powers & Tupper (1974)
−23	50–200	Anode-break response; calcium inward current under voltage clamp	Okamoto *et al.* (1977)
−14	—	—	Fulton & Whittingham (1978)
−7	39	Anode-break response	Yoshida (1983)
−18	11	—	Eusebi & Siracusa (1983)
−22	11	Anode-break response	Eusebi, Colonna & Mangia (1983)
−35	96	—	Jaffe, Sharp & Wolf (1983)
−35	140	—	Igusa, Miyazaki & Yamashita (1983)
−91	430	Action potential	Present study
		Hamster eggs	
−29	150	Anode-break response	Miyazaki & Igusa (1982)
−9 to −100	14–440	Anode-break response	Georgiou *et al.* (1983)
−22	19	—	Eusebi & Siracusa (1983)
−97	410	Action potential	Present study
		Rabbit eggs	
−71	10	—	McCulloh, Rexroad & Levitan (1983)

* Determined from the linear part of the current/voltage relation. Hamster and mouse eggs show rectification above 0 mV and also below −150 mV as found by Miyazaki & Igusa (1982) in cells without action potentials. In the present study the current/voltage relation was linear in the range −70 to −150 mV.

Mangia, 1983; Yoshida, 1983) and voltage-clamp experiments (Okamoto *et al.* 1977; Yamashita, 1982). Calcium channels also occur in the eggs of the starfish and sea urchin (Miyazaki *et al.* 1975; Hagiwara, Ozawa & Sand, 1975; Jaffe, 1976; Chambers & de Armendi, 1979).

Changes in the sodium, potassium, chloride or calcium concentrations in the bathing solution influenced the egg's membrane potential in ways suggesting that the membrane is permeable to these ions. The high value of the egg's membrane resistance, however, indicates that the absolute magnitudes of ionic permeabilities must be low (cf. Powers & Tupper, 1975). It is interesting to note that unfertilized sea urchin eggs also have high resistances (*ca.* 500 MΩ) and that potassium, the most permeant ion in these cells, has a permeability as low as 10^{-8} cm . s^{-1} (Jaffe & Robinson, 1978).

Several features of the action potentials in mouse and hamster eggs deserve comment.

The threshold for excitation is about −50 mV which is about 40 mV more positive than the membrane potential recorded in eggs bathed in normal solution. Fluid in the mouse oviduct, however, contains a higher concentration of potassium, i.e. 25 mM, than that present in our normal solution (Borland, Hazra, Biggers & Lechene, 1977). Our measurements of the effect of extracellular potassium on the membane potential indicate that the membrane potential of eggs in oviducal fluid might be about 20 mV more negative than the threshold potential. Moreover, eggs were excitable in solutions with high potassium concentrations.

The maximum rate of change of membrane potential during the upstroke of the action potential was about 3 V . s^{-1}. This rate is proportional to the positive ionic current entering the egg. The absolute value of this current can be estimated roughly since the membrane

capacitance of the egg has been determined as 1·4 μF . cm^{-2}. So the ionic current entering the egg during the upstroke of the action potential was about 4·2 μA . cm^{-2}. Since the egg's membrane area is apparently about 2×10^{-4} cm^2 the total inward current into the egg was about 0·84 nA. This value is in rough agreement with the size of the inward current produced by depolarizing command pulses in previous voltage-clamp experiments on mouse eggs (Okamoto *et al.* 1977).

In some records (Figs. 3 and 4) the action potential did not overshoot zero. A possibility is that these negative peak values reflect the presence of tip potentials at the recording micro-electrode. However, no such tip potentials were observed on removing the micro-electrode from an egg with an undershooting action potential. Moreover, we recorded large membrane potentials in some eggs and yet these also had overshooting action potentials (Table 3). Although the peak value of the action potential was usually positive it was substantially less than the expected equilibrium potential for calcium (E_{Ca}) as found also in other cells with calcium action potentials (Reuter, 1973). This casts doubt on the strength of the evidence for calcium channels based on a linear relation between the peak of the action potential and the log of extracellular calcium concentration. An alternative view is that the peak of the action potential is more negative than E_{Ca} because an outward positive current occurs as suggested first by Fatt & Ginsborg (1958) for crustacean muscle. It was considered that potassium ions carried this outward current in crustacean muscle. The ionic movements during the action potential in mammalian eggs remain to be investigated in detail. The present electrical evidence suggests the presence of a calcium influx during the upstroke of the action potential.

Does micro-electrode insertion mimic sperm entry?

The progressive rise in potential and resistance which was evident in intracellular recordings from hamster eggs (Georgiou *et al.* 1983) and in the present records from mouse eggs (Fig. 1) might be due to processes initiated by electrode insertion itself rather than to improved micro-electrode sealing as suggested above. Could it be that the insertion of a micro-electrode, in fact, mimics the entry of sperm and that the rise in potential and resistance reflects the process of activation of the egg? This question has been addressed in the literature on eggs of invertebrates which also show a rise in potential and resistance after cellular impalement (e.g. Chambers & de Armendi, 1979). The available evidence based on intracellular recordings from unfertilized and fertilized eggs, on unidirectional flux measurements in unfertilized eggs and on extracellular recordings of 'action currents' at fertilization indicates that micro-electrode insertion does not mimic sperm entry (Jaffe & Robinson, 1978; Chambers & de Armendi, 1979; Whitaker & Steinhardt, 1982, 1983). Unfortunately the corresponding evidence for mammalian eggs is sparse and equivocal.

Powers & Tupper (1975) measured unidirectional fluxes of sodium, potassium and chloride ions in unfertilized mouse eggs. Assuming low values of the membrane potential they estimated ionic permeabilities and hence ionic conductances. The estimated total ionic conductance was 880 μS . cm^{-2} whereas the measured membrane conductance was 380 μS . cm^{-2} (Powers & Tupper, 1974). The data in this paper give a membrane conductance of about 10 μS . cm^{-2}. Thus there exists a serious disparity between the ion flux determinations and our electrical measurements. The disparity needs experimental investigation and at present it excludes any useful argument about the size of the potential and resistance of eggs on the basis of their ionic fluxes.

It is crucial to establish the potential and resistance of fertilized eggs. It is our contention, however, that all previous recordings from fertilized mammalian eggs probably suffered from impalement-leak artifacts. In hamster eggs fertilization is accompanied by a series

of transient hyperpolarizations and transient rises in conductance (Miyazaki & Igusa, 1981, 1982; Igusa & Miyazaki, 1983). In mouse eggs fertilization is accompanied by a small hyperpolarization (Igusa, Miyazaki & Yamashita, 1983; Jaffe, Sharp & Wolf, 1983) and in rabbit eggs by a slow depolarization and repetitive diphasic potential changes (McCulloch, Rexroad & Levitan, 1983). Since no investigator has reported a rise in resistance accompanying fertilization it seems highly unlikely that the changes we have reported in the electrical properties of hamster and mouse eggs upon impalement reflect an activation process started by micro-electrode penetration. Moreover, when a second micro-electrode is inserted into a hamster or mouse egg after the high potential and resistance state is attained there is always a transient fall both in the potential and the resistance of the egg (Fig. 4, Georgiou *et al.* 1983; Fig. 1, present study) as would be expected for a transient leak pathway at the micro-electrode tip.

Finally, strong indirect evidence that insertion of a recording micro-electrode cannot activate mammalian eggs comes from a detailed study by Uehara & Yanagimachi (1977). These authors activated hamster eggs by piercing them with fine glass needles. Small needles with diameters less than 2 μm were ineffective at causing activation whereas needles with diameters in the range 3–5 μm were effective provided several piercing movements were made. Since the piercing movement amounted to pushing the needle through the egg till it emerged at the other side it is highly unlikely that the relatively gentle insertion of a fine-tipped micro-electrode can activate mammalian eggs. Moreover, Fulton & Whittingham (1978) have reported that insertion of micropipettes with fine tips ($\ll 0{\cdot}5$ μm) did not activate mouse eggs.

Function of calcium action potential

It is possible that sperm entry into a mammalian egg evokes an action potential since a similar finding has been made in the eggs of sea urchins (Jaffe, 1976; Chambers & de Armendi, 1979). In sea urchins, however, it is by itself not a sufficient stimulus for egg activation and indeed activation can occur in the absence of action potentials (see Whitaker & Steinhardt, 1982). An action potential occurring during the fertilization of a mammalian egg might serve as an appropriate trigger for exocytosis of the material in the cortical granules by virtue of the calcium influx which must occur. It is doubtful whether sufficient calcium will flow in during an action potential to produce a widespread rise in the cytosolic calcium concentration. However, it is possible that the rise in cytosolic calcium concentration arising from an action potential could cause secondary calcium release from intracellular stores (Gilkey, Jaffe, Ridgway & Reynolds, 1978). Evidence from studies with aequorin suggests that fertilization of mouse eggs is accompanied by periodic rises in the cytosolic calcium concentration (Cuthbertson, Whittingham & Cobbold, 1981). Moreover, Fulton & Whittingham (1978) have shown that calcium ionophoretically injected into mouse eggs causes parthenogenetic activation. The possible roles that the action potential plays in the processes of the cortical reaction and the polyspermy block remain to be investigated and the present paper describes results suggesting that such investigations could be worthwhile.

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Single-channel currents in unfertilized mouse eggs

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The presence of calcium channels in unfertilized mouse eggs is indicated by calcium action potentials evoked by depolarization (Georgiou, Bountra, Bland & House, 1984) and by inward calcium currents in voltage clamp experiments (Okamoto, Takahashi & Yamashita, 1977).

In order to examine these channels further, cell-attached patch recordings (Hamill, Marty, Neher, Sakmann & Sigworth, 1981) have been made from zona free mouse eggs. To facilitate giga-seal formation and to enhance the mean amplitude of single-channel currents, the solution bathing the egg and filling the patch electrode contained 80 mM-$SrCl_2$ as a replacement for NaCl. When the patch electrode was held near to 0 mV (transpatch potential is then about −90 mV), spontaneous inward currents were observed (Fig. 1).

Fig. 1. Inward currents at a patch potential 12 mV more negative than the cell's resting membrane potential (filtered at −3 dB 100 Hz). The egg was bathed at 24 °C in a solution (pH 7·2) containing (mM): $SrCl_2$, 80; KCl, 5; $CaCl_2$, 1; $MgCl_2$, 1·2; glucose, 5·6; lactate, 20; buffered using Hepes, 5, and NaOH, 2·5. Methods of obtaining zona free eggs have been described by Georgiou *et al.* (1984).

At rest the channels were seen to open in bursts, as has been found in other preparations containing calcium channels (Reuter, 1983). The mean ± S.D. values of the amplitude of these channel currents were found to be 1·3 ± 0·5 pA ($n = 342$); probability of opening was 0·18. Furthermore channels open in synchrony with evoked action potentials.

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Micro-electrode recordings from rabbit ciliary epithelium *in vitro*

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The ciliary epithelium consists of a pigmented cell layer facing the stroma of the ciliary body and an adjacent non-pigmented layer facing the aqueous humour. Little is known about the electrophysiology of the cells of this bilayer, which participates in the formation of the aqueous humour (Berggren, 1960; Miller & Constant, 1960). A preliminary study has been made on isolated ciliary processes from adult albino New Zealand rabbits. A single process was placed in a chamber on an inverted microscope and superfused with a solution (pH 7·4) at 27 °C containing (mM): NaCl, 123; KCl, 4·7; $CaCl_2$, 2·5; $MgCl_2$, 1·2; $NaHCO_3$, 25; glucose, 25. The solution was equilibrated with air and its pH did not change during experiments. A micro-electrode (50 MΩ) filled with 2 M potassium acetate was inserted into the ciliary epithelium across the basal surface of the non-pigmented layer. The mean (±S.D.) values of the recorded potential and input resistance were -65 ± 15 mV ($n = 77$) and 37 ± 28 MΩ ($n = 17$). Lucifer Yellow CH dye dissolved in 1 M-LiCl was ionophoresed from a micro-electrode by hyperpolarizing current pulses for 10–30 min during some potential measurements; the dye marked the impaled cell and adjacent cells in the non-pigmented and pigmented layers. Evidence of electrical coupling between cells was also obtained. When a current pulse was passed through an intracellular micro electrode, an electrotonic potential could be recorded by a second intracellular electrode placed up to 300 μm (*ca.* 20 cell diameters) from the first. Epithelial cells were depolarized by raising the extracellular concentration of potassium whereas large changes in the extracellular concentrations of sodium, chloride or bicarbonate ions caused small and variable alterations of the membrane potential. The relation between membrane potential and log $[K]_o$ was linear with a mean slope of 38 mV (16 cells). During a period (10–30 min) Of deprivation of extracellular potassium the cells hyperpolarized by about 5 mV. When potassium was restored to the bathing solution the cells transiently hyperpolarized by about 10 mV (mean ± S.D. = 12 ± 6 mV, $n = 18$) without a conspicuous change in input resistance. This response to potassium restoration was abolished by ouabain (10^{-5} M) or by bathing the epithelium in a solution lacking sodium. It was concluded that ciliary epithelial cells are relatively permeable to potassium, that the cells probably have an electrogenic Na/K pump and that the non-pigmented and pigmented cell layers are coupled.

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An Electrophysiologic Study of Rabbit Ciliary Epithelium

Keith Green,*‡ Chas Bountra,† Panos Georgiou,† and C. Randall House†

Microelectrode recordings from cells in rabbit ciliary epithelium have been made in vitro. Ionophoresis of Lucifer Yellow dye from microelectrodes during measurements of potential confirmed that the recordings were intracellular. Dye passed from the impaled cells into adjacent cells in both the nonpigmented and pigmented layers of the epithelium. Electrical coupling between epithelial cells also was observed. The mean (±SD) values of the potential measured across the basolateral membranes of the nonpigmented cells was −65 ± −15 mV (n = 77); the mean value of the input resistance at this intracellular recording site was 37 ± 28 MΩ (n = 17). The membrane potential was reduced by raising the concentration of extracellular potassium but unaffected by changes in the concentrations of sodium, chloride, or bicarbonate ions. After a period of deprivation of extracellular potassium, the cells hyperpolarized without a measurable change in membrane resistance when potassium was restored to the bathing solution; this transient response to potassium was abolished by preincubation with ouabain or by bathing the epithelium in a solution lacking sodium. It was concluded that the ciliary epithelial cells are permeable to potassium but exhibit only a low permeability to sodium, chloride, or bicarbonate ions; that the cells possess an electrogenic Na/K pump; and finally, that all of the cells in the epithelium function as a syncytium. Invest Ophthalmol Vis Sci 26:371–381, 1985

The mammalian ciliary epithelium consists of a nonpigmented cell layer facing the aqueous humor and an underlying pigmented layer facing the stroma of the ciliary body. The cells of each layer are oriented such that the apical surfaces of the nonpigmented cells adjoin the apical surfaces of the pigmented layer. This bilayer forms the aqueous humor[1] by ultrafiltration and active secretion,[2,3] although a detailed description is not yet available. Several studies of the transport properties of the rabbit's iris-ciliary body have been made in vitro.[3–8] By contrast, studies in vivo are not preferred because they are technically difficult and subject to inflammatory reactions induced by prostaglandin release in the eye.

Three reports of microelectrode recordings from rabbit ciliary epithelium have been made,[9–11] but no evidence was provided by the authors to confirm that the recordings were genuinely intracellular. We have undertaken an initial electrophysiologic study of the rabbit ciliary epithelium to provide data on the value of the intracellular resting potential of both cell layers, the existence of electrical coupling between cells and the influence of ionic changes on the resting potential. This study constitutes a first attempt in a new approach to study the cellular electrophysiologic properties of this tissue.

A preliminary report has been presented to the Physiological Society.

Materials and Methods

Animals in this study were used in accordance with the ARVO Resolution on the Use of Animals in Research. Adult albino New Zealand rabbits were killed with a blow to the back of the neck. The anterior segments of both eyes were removed quickly and placed in a dish containing a modified Krebs bicarbonate Ringer solution.[12] An iris-ciliary body was dissected from the anterior uvea as previously described.[3,13,14] The isolated iris-ciliary body was pinned on to a layer of Sylgard at the bottom of a Perspex dish by four pins inserted into the choroid attached to the periphery of the preparation. A single ciliary process was dissected from the iris and transferred to a 4-ml chamber in which two monofilament

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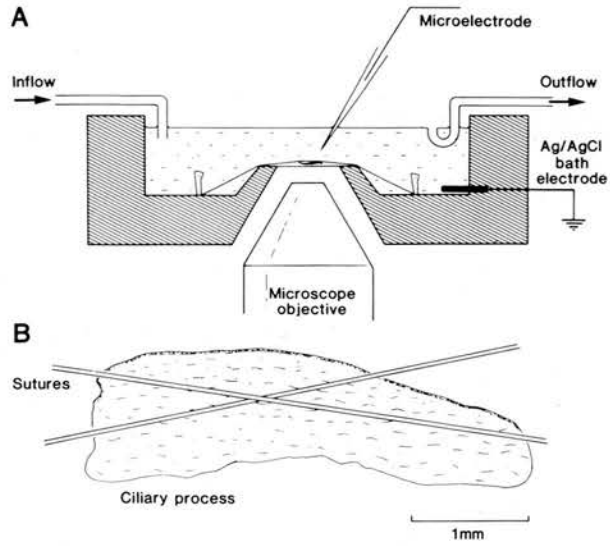

Fig. 1. Experimental arrangement for recording potential in an isolated ciliary process. **A**, Diagram of chamber on stage of inverted microscope. **B**, Plan of isolated ciliary process held under monofilament sutures.

sutures (9-0, Ethicon) were placed in a cross (Fig. 1). The isolated process was held under the crossed sutures in order to prevent movement caused by the continuous flow of the solution (27°C) at 1 ml/min through the chamber. This flow rate was selected in order to minimize tissue disturbance during perfusion, thereby allowing increased impalement times.

Intracellular Recording

The chamber was placed on the stage of an inverted microscope (Biovert; Reichert, Austria) and interference contrast optics at either ×40, for initial orientation, or at ×160 for microelectrode impalement. The potential was recorded between a microelectrode (40–70 MΩ), filled with 2 M potassium or sodium acetate, and a solid Ag/AgCl electrode in contact with the solution. The possibility that potassium leakage from an intracellular electrode alters the cell's potassium concentration and potential seems slight because steady potentials were recorded for long periods (30–120 min). Presumably a potassium concentration gradient would be dissipated by diffusion through the intercellular junctions (see *Results*). In experiments where the chloride concentration of the bathing solution was changed, the bath was earthed via a 3 M KCl saline-agar bridge. The microelectrode was connected to the input of a high impedance pre-amplifier (Model KS 700; WP Instruments, Inc., New Haven, CT), the probe head of the amplifier was held in a sliding manipulator (Carl Zeiss; Jena, East Germany). In some experiments, current pulses from a Devices stimulator triggered by a Digitimer (D4030; Devices Ltd.) were passed via a bridge circuit between the barrel of the microelectrode and the bath electrode. The bridge was balanced during the passage of current pulse (0.25 nA, 400 msec) before microelectrode penetration. Cellular impalement was achieved by

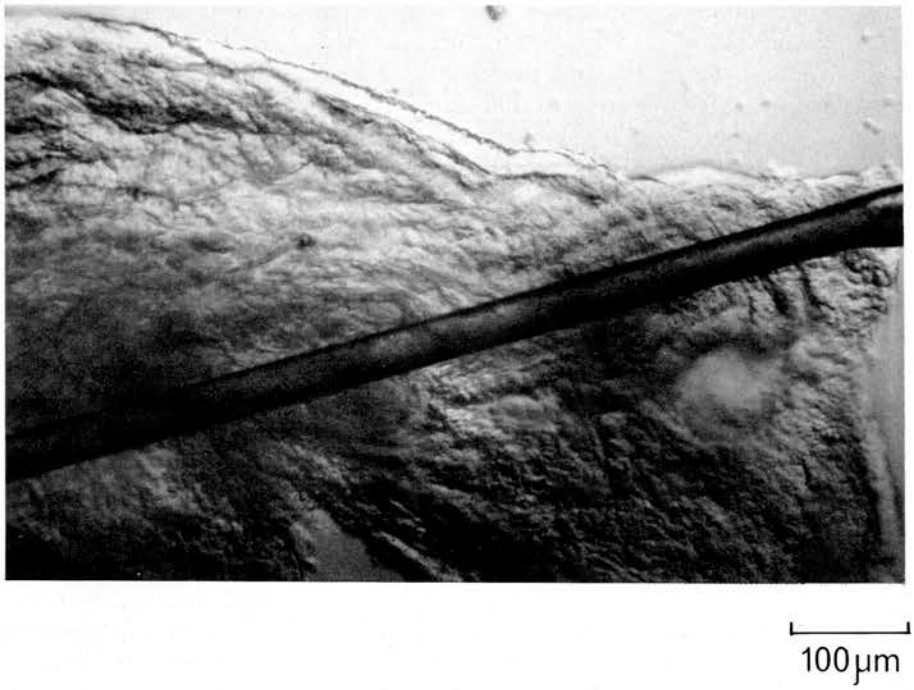

Fig. 1. C, Light microscope picture of region of isolated process mounted in experimental chamber.

resting the microelectrode tip on the surface of the ciliary epithelium and increasing the negative capacity compensation applied to the microelectrode so that it went briefly into electrical oscillation. After impalement when the potential had attained a steady value, the bridge balance was checked with passage of a current pulse (0.25 nA, 100 msec); frequent rebalancing was not required. Impalement acceptance depended upon achieving a rapid deflection of the recording, which either was maintained immediately or slowly increased, presumably due to sealing of the membrane around the electrode, over at least 10 min. It was not possible to obtain intracellular recordings with the lens capsule left in situ due to the rigid nature of the capsule and suspensory ligaments either preventing penetration into cells or by breaking of electrodes during attempts at impalement. This is presumably due to the convoluted nature of the tissue with many infoldings preventing microelectrode impalement.

For dye-marking experiments, microelectrodes were filled with Lucifer Yellow CH (30 mg/ml) and hyperpolarizing current pulses (10 nA, 500 msec) were passed at 1 Hz through the electrode for 10–30 min to ionophorese dye into the impaled cells. Successful impalements were judged by (1) the membrane potential per se, at about −80 mV, and (2) maintenance of cellular potential during dye ionophoresis. The latter was checked periodically and discarded if the cellular potential was less than 15% of its original, pre-ionophoresis, value. Lucifer Yellow was made up in 1 M LiCl solution, which was deposited at the electrode tip and the electrode was back-filled with 1 M LiCl.[15] Tissues were fixed in 10% Formol saline and subsequently embedded in wax. Unstained serial 5-μm sections were examined under ultraviolet light and photographed; subsequently, the same sections were stained with hematoxylin-eosin and rephotographed to delineate the nonpigmented and pigmented cells; the latter cells lacked pigment since tissues were obtained from albino rabbits.

To determine the extent, if any, of electrical coupling between epithelial cells, two microelectrodes were inserted into the preparation at a known distance apart. A rectangular current pulse was passed through one microelectrode, and the electrotonic potentials monitored by both electrodes were recorded. The effect of varying the distance between the microelectrodes on the size of the electrotonic potential recorded by the second microelectrode was examined. The procedure for carrying out the electrical coupling measurements involved the following steps: (1) insert first microelectrode for current injection; (2) record intracellular potential of first electrode; (3) insert second microelectrode for monitoring electrotonic potentials; (4) record intracellular potential of second electrode; (5) pass rectangular current pulse through first electrode and record electrotonic potentials with both electrodes; (6) measure distance between electrode insertion sites with filar micrometer eyepiece; and (7) withdraw second electrode and repeat steps 3–7 at new site.

Permanent experimental records were obtained as pen recorder traces on a Devices M2 Recorder or as photographs of the screen of a storage oscilloscope (RM5113; Tektronix Ltd., Portland, OR).

Solutions

The modified Krebs Ringer bicarbonate solution contained (mM): NaCl, 123; KCl, 4.7; $CaCl_2$, 2.5; $MgCl_2$, 1.2; $NaHCO_3$, 25; glucose 25 mM. Variations of composition were: K-free solution in which KCl was omitted; low Na solution in which choline chloride replaced NaCl on an equimolar basis; Na-free solution in which choline chloride replaced NaCl and choline bicarbonate replaced $NaHCO_3$; Cl-free solution in which sulphate replaced chloride, the calcium concentration was 2 mM, and the osmolarity was adjusted with sucrose; KCl-free solution, a modified form of Cl-free solution in which Na_2SO_4 replaced K_2SO_4; HCO_3-free solution in which Na propionate replaced $NaHCO_3$. Solutions also were used in which the [K][Cl] product was constant; these solutions had appropriate adjustment of NaCl and sucrose to keep the osmolarity constant. The pH of all solutions lay in the range 7.4–7.8, and the osmolarity was about 305 mosm/l.

In some experiments, ouabain (Strophanthin-G; Sigma Chemical Co.) was added to solutions to give a final concentration of 1, 0.1, or 0.01 mM.

Results

Intracellular Recording

In this study, microelectrodes were inserted in the ciliary epithelium across the basal surface of the nonpigmented cell layer. The insertion of a microelectrode was achieved by a brief period of electrical oscillation (see *Methods*) rather than by mechanical displacement. Lucifer Yellow ionophoresed from microelectrode tips during stable potential recordings was found within epithelial cells upon subsequent histologic examination (see below). During intracellular recording, the potential normally attained a steady value several minutes after impalement. Only values of the potential that remained constant (Δ < 1 mV change) for at least a further 10 min were

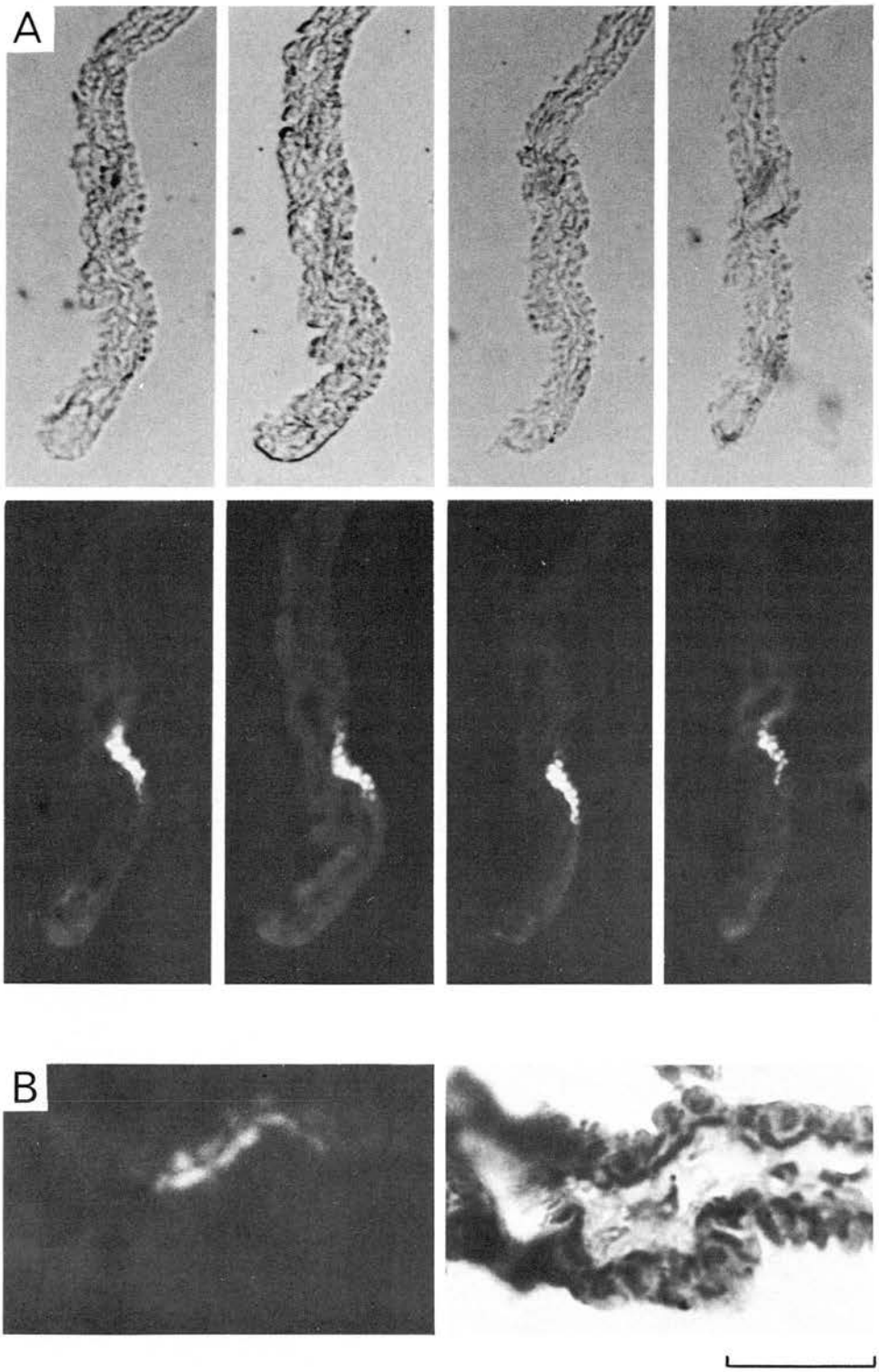

Fig. 2. Light and ultraviolet pictures of sections of an isolated ciliary process, which has been labelled by ionophoresis of Lucifer Yellow from an intracellular microelectrode. Fluorescent staining was found in eight adjacent sections. **A**, Light and corresponding ultraviolet pictures of second, fourth, fifth, and seventh sections from the series. After photography, the sections were stained with hematoxylin-eosin and rephotographed on the light microscope. **B**, Light and ultraviolet pictures of dye-labelled region of the eighth section showing that the Lucifer Yellow occurs in both nonpigmented and pigmented cells; this section has been stained with hematoxylin-eosin to show both cell types, nonpigmented and pigmented. The calibration bar indicates 90 μm for **A** and 30 μm for **B**. Evidently there has been some tissue shrinkage during the tissue processing. The membrane potential of the impaled cell was −88 mV and the cell was labelled by passing 10 nA pulses (500 msec) at 1 Hz for 11 min.

accepted. The input resistance did not change during the course of measurements. The mean ($\pm$SD)potential difference was -65 ± 15 mV for 77 impalements in 74 isolated processes from 26 rabbits; the distribution of values was normal. This value for intracellular potential represents impalements of both nonpigmented and pigmented epithelial cells using either potassium or sodium acetate-filled microelectrodes: no changes occurred in the cellular potential despite recording of membrane potential for up to 2 hr, thus

flux of microelectrode contents into the cells did not appear to introduce any artifacts. It was difficult to be sure of exactly which cell layer was impaled securely, although on several occasions a transient fall in potential was noted as advancement of the electrode tip was made before re-entering another zone of equal potential. In addition, as results below indicate, dye ionophoresed into one cell passes between layers; thus, it is difficult to be sure exactly which layer has been penetrated. All values in this sample were taken from microelectrode recordings that remained stable for more than 10 min. Often it was possible to record the membrane potential for periods exceeding 30 min. All potential records in this paper are labelled with the time of the recording, zero-time being taken at the moment of impalement.

Cell Coupling

Dye ionophoresed from a microelectrode during a period of stable potential recording stained not only the impaled cell but also a group of its neighbors (Fig. 2A). Presumably, the dye passed from the impaled cell through permeable junctions to adjacent cells as found in other epithelial tissues showing electrical coupling.[16–18] Apparently dye also passed between nonpigmented and pigmented cells in adjacent layers and seemed to be retained preferentially in the nuclei (Fig. 2B).

Evidence of electrical coupling between cells also was obtained in four experiments. When a rectangular current pulse was passed through an intracellular electrode, an electrotonic potential could be recorded by a second intracellular microelectrode placed up to 300 μm away. The results of a representative experiment are shown in Figure 3. Each point represents a single measurement of the amplitude of the electrotonic potential, and the points are joined by straight lines for illustrative purposes. Another feature of the intracellular recordings from ciliary epithelial cells, which indirectly supports the idea that they are coupled electrically, emerged from measurements of the current/voltage relations of these cells.

Current/Voltage Relations

Rectangular current pulses passed through an intracellular electrode produced graded electrotonic potentials. The relations between current and potential change were linear for small displacements of the membrane potential (Fig. 4). The mean ($\pm$SD)input resistance obtained from such linear plots was 37 $\pm$ 28 MΩ (17 cells). A few values were well below 1 MΩ and have not been included in the sample of measurements. Due to cell coupling, the value for

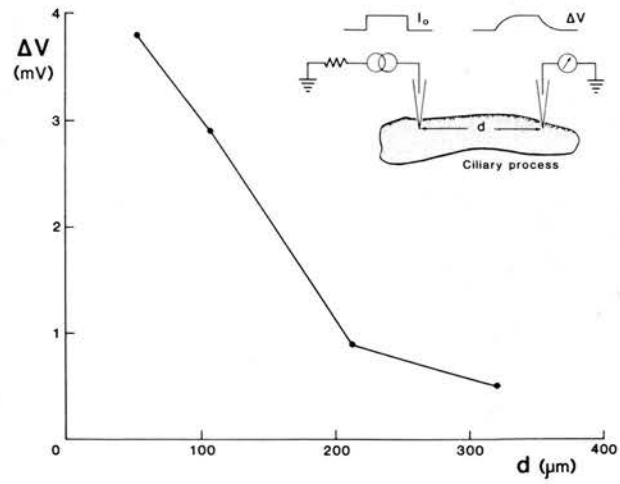

Fig. 3. Typical plot of ΔV against distance (μm) for electrotonic potentials measured in one electrode relative to another electrode. The experimental arrangement is shown in the upper right inset. A current passed through the first intracellular electrode (for current injection) did not produce a conspicuous electrotonic potential at the second microelectrode before it was inserted into a cell.

input resistance is an underestimate, since injected current is dissipated laterally as well as across the cellular membrane. Indeed, the time course of the charging transient and the low input resistance are indicative of more cells being charged than just the impaled cell. The epithelial cells in the ciliary processes are small (ca. $20 \times 10 \times 10$ μm) and with a given density of ion channels per unit area, a cell will have a greater input resistance as its size decreases. Assuming a specific membrane resistance[19] of 4000 Ωcm^2 and a cell area of 10^{-5} cm^2, the input resistance of an isolated ciliary epithelial cell would be 400 MΩ.

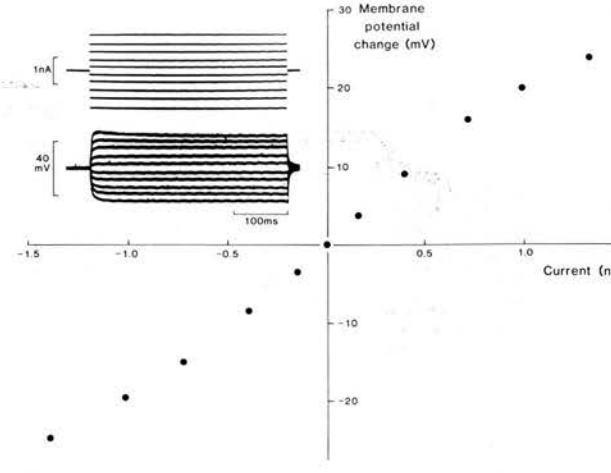

Fig. 4. Plot of voltage change, produced by passing current pulses through a microelectrode inserted in a ciliary epithelial cell, against the amplitude of the current pulse. The current pulses and the corresponding electrotonic potentials are shown in the inset.

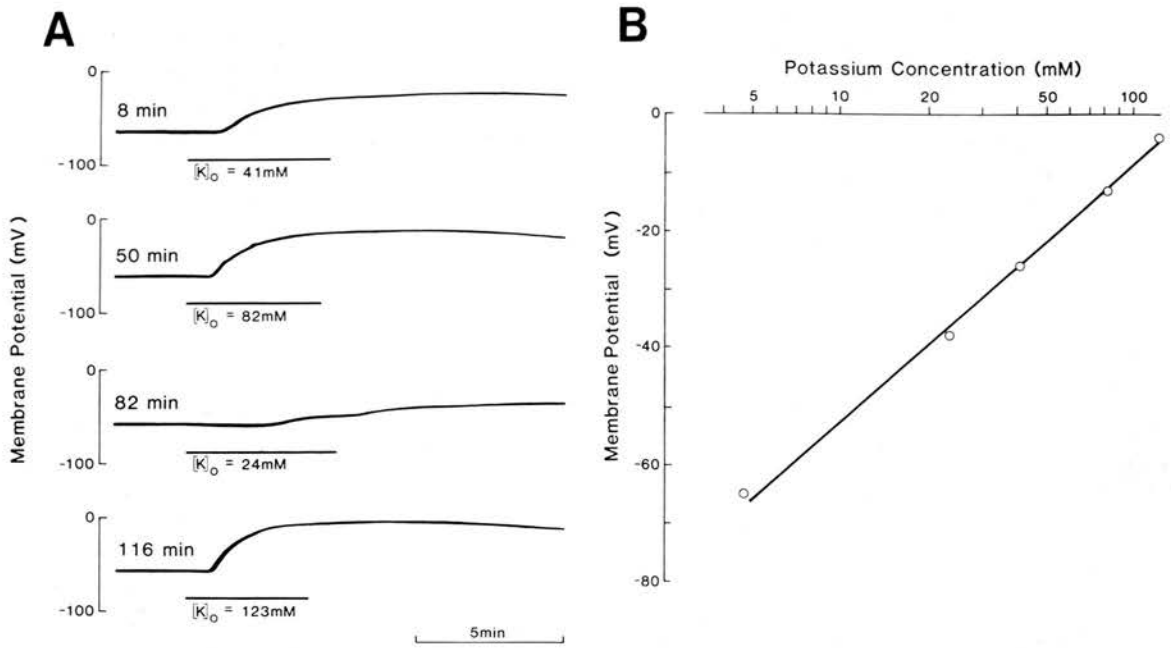

Fig. 5. Effect of extracellular potassium on the membrane potential of a ciliary epithelial cell. **A**, Responses of membrane potential to elevations of the potassium concentration in the bathing solution. In this and all subsequent figures, the time labelling on traces indicates the time after cellular impalement. The time courses of the changes in potential reflect the mixing time in the experimental chamber at the chosen rate of superfusion of the tissue. **B**, A graph of membrane potential against logarithm of $[K]_0$ for the results shown in **A**. The horizontal bars indicate the period of solution change.

Such high input resistances were not observed. The low values found are similar to those recorded in epithelia with extensive electrical coupling.[20] For a nonpigmented cell with a surface area of 10^{-5} cm^2 and resistance of 40 MΩ, the time constant would be about 0.4 msec. However, the time constant of the electrotonic potential was about 5 msec. Thus, the relatively low input resistance and long time constant suggest that the small nonpigmented cells are coupled electrically.

Table 1. Effect of extracellular potassium concentration on the membrane potential of ciliary epithelial cells

$[K]_o$ *(mM)*	*Depolarization produced (mV)*
9	8 at constant
9	5 $[K]_0[Cl]_0$
10	7
24	19
30	15
30	22
41	22
41	29
60	44
82	48
123	49 ± 9*
125	55 at constant
125	52 $[K]_0$ $[Cl]_0$
125	48

Each value derived from a separate cell.
* Mean ± SD (nine cells).

Ionic Substitutions

To establish which of the main extracellular ions influences the membrane potential of ciliary epithelial cells, a number of ionic substitutions were made in the bathing solution.

Potassium: Bathing an isolated ciliary process in K-free solution always led to a sustained hyperpolarization of about 5 mV below the potential noted in Krebs bicarbonate Ringer solution. The mean (±SD) value for the sustained hyperpolarization in K-free solution was 6 ± 5 mV (20 cells). An increase in the potassium concentration, $[K]_0$, caused depolarization that was graded with the size of the change in $[K]_0$ (Fig. 5). It should be noted that the potential responses to $[K^+]_0$ were slow due to the relatively slow perfusion rate (see *Methods*). The strong dependence of potential on $[K]_0$ suggests that the cell membrane is permeable to potassium ions. This was confirmed in a separate series of experiments where $[K]_0$ was increased while the product $[K]_0[Cl]_0$ remained constant in order to determine whether or not the response to K^+ was Cl^--dependent. A graded depolarization with increased

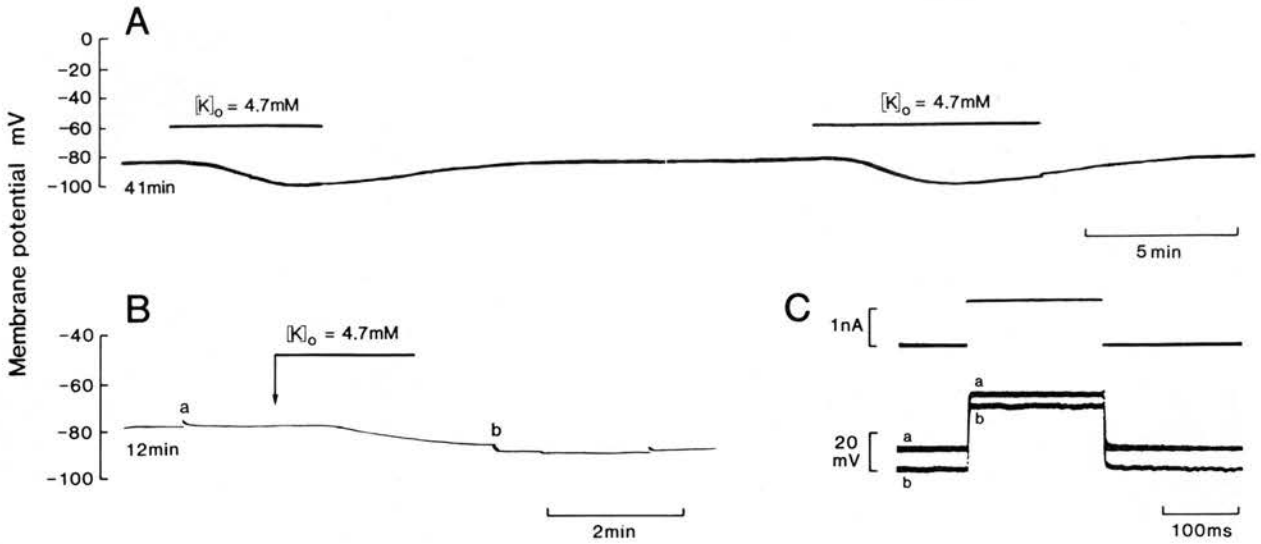

Fig. 6. Potassium readmission responses recorded in a ciliary epithelial cell. The horizontal bars indicate the periods when the ciliary process was bathed in Krebs bicarbonate Ringer containing 4.7 mM potassium; at other times, the tissue was bathed in a K free solution. The time labelling on the trace indicates the time after cellular impalement. The intracellular recording shows two responses (**A** and **B**) taken from a series of four readmission responses to potassium. **C** indicates the membrane resistance measured at points a and b in Part **B**; note the absence of a change in resistance.

$[K]_0$ was observed again (Table 1) in general accord with the results illustrated in Figure 5 obtained at a fixed chloride concentration.

Other ions: Changes in the concentrations of sodium, chloride or bicarbonate ions in the bathing solution failed to change the membrane potentials of ciliary epithelial cells. The compositions of the solutions used in these experiments are described in *Methods.*

Potassium Readmission Response

It was found that, after a period of superfusion with K-free solution for at least 10 min, the readmission of Krebs bicarbonate Ringer solution always caused a transient hyperpolarization of about 10 mV lasting about 10 min. The slow time course of the response probably reflects the kinetics of mixing in the chamber. The mean (±SD) value of the hyperpolarization caused by readmission of the normal potassium concentration (4.7 mM) to K-free solution was 12 ±6 mV (18 cells). In many cells, it was possible to repeat the potassium deprivation–readmission cycle several times and obtain consistent hyperpolarizing responses. An example shows two potassium readmission responses from a series of four responses, which had amplitudes of 16, 15, 17, and 20 mV (Fig. 6). Also shown in Figure 6 is an example of the lack of change in input resistance following readmission of potassium, although the small change expected from a change from K_0 = 0 to K_0 = 4.7 might not be seen easily due to current dissipation in the syncytium.

Readmission responses also were evoked by potassium concentrations above that in Krebs bicarbonate Ringer solution (four experiments) as observed previously in liver cells.[21] It was notable that readmission of a solution containing $[K]_0$ = 100 mM after superfusion with K-free solution caused a marked hyperpolarization, which lasted for about 2 min and was overtaken by a large characteristic depolarization (Fig. 7).

The potassium readmission response did not occur in ciliary processes that had been bathed in Na-free solution before and during potassium deprivation.

Effect of Ouabain

Ciliary processes were bathed in Krebs bicarbonate Ringer solution containing various concentrations of ouabain to examine whether the membrane potential has an electrogenic component. Ouabain at 0.01 mM did not change the potential of cells. When ouabain was applied at 0.1 or 1 mM during fast superfusion, it did not cause a rapid change of potential but led to a slow depolarization, which could not be reversed by superfusion with Krebs bicarbonate Ringer solution or Ringer solution with raised potassium concentration (10 mM). Even a brief exposure (2 min) to 0.1 mM ouabain was sufficient to induce a prolonged and irreversible depolarization (Fig. 8).

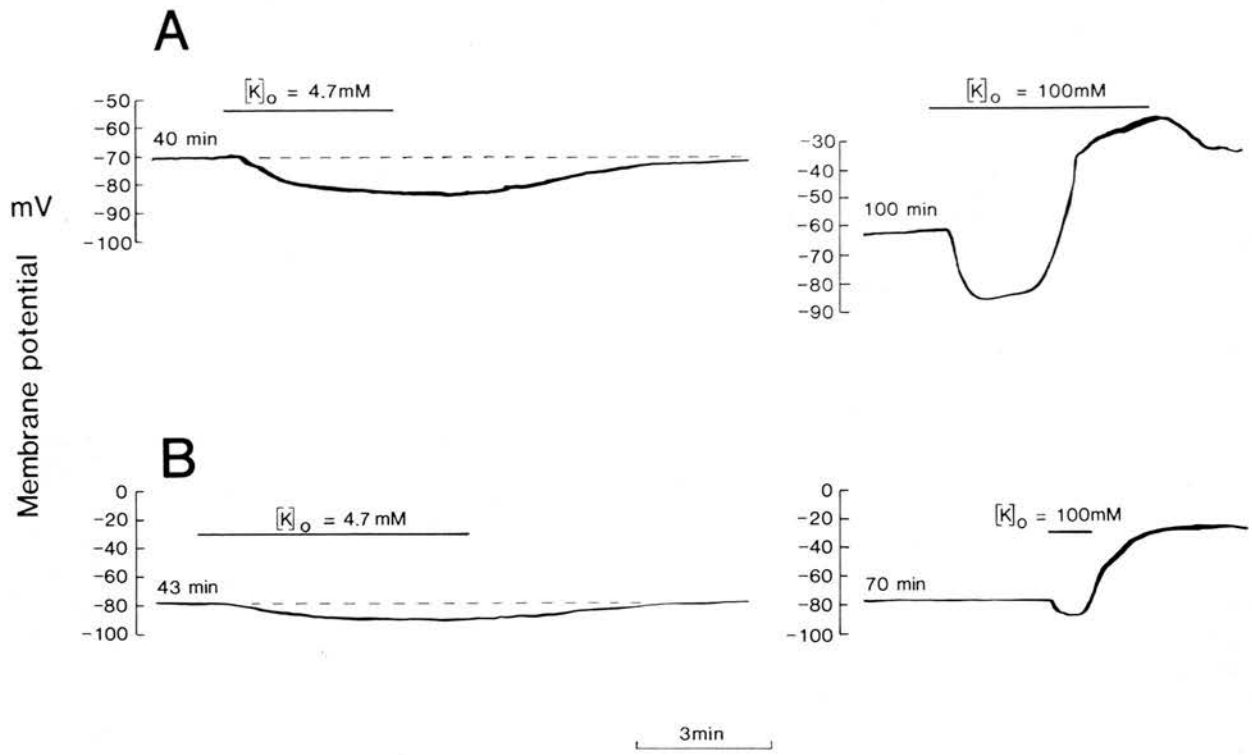

Fig. 7. Two examples of the readmission responses to potassium. **A**, Intracellular recording from a ciliary epithelial cell showing responses to potassium at 4.7 mM and 100 mM in the bathing solution. The high concentration of potassium was obtained by replacing part of sodium composition of the solution. **B**, Similar readmission responses recorded from a cell in another ciliary process. The time labelling on the trace indicates the time after cellular impalement.

Despite the failure of ouabain to reveal an electrogenic component of the membrane potential of cells, it was of interest to observe its effects on the potassium readmission response. Ciliary processes were superfused with K-free solution for periods of at least 20 min and then exposed for 2 min to a K-free solution containing 0.01 mM ouabain after a normal potassium readmission response had been recorded (Fig. 9). Evidently ouabain blocked the hyperpolarizing responses to potassium (readmitted at 4.7 mM or 100 mM) and the observed depolarizations were consistent with the cell's passive permeability to potassium ions.

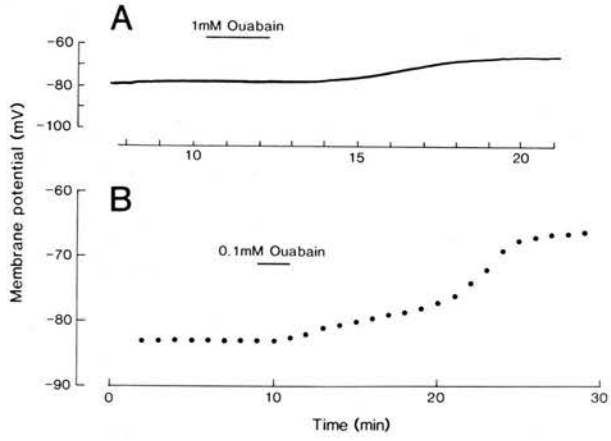

Fig. 8. Effect of ouabain on the membrane potential of ciliary epithelial cells. Recordings from cells in different ciliary processes are illustrated in **A** and **B**. The data in **B** have been represented graphically to illustrate the prolonged time course of the depolarization induced by ouabain.

Discussion

The results of this initial study of rabbit ciliary epithelium are at variance with those of Miller and Constant,[10] Berggren,[9] and Cole[11] who reported that the nonpigmented, and pigmented cells had membrane potentials of −30 mV and −60 mV, respectively. No evidence for a group of low-potential cells has been found in our work. The difference undoubtedly reflects the improvements in microelectrode recording techniques that have occurred since the 1960s. Previous recordings were made with low resistance microelectrodes (10–20 MΩ), and probably they caused cellular damage and depolarization. We found it impossible to obtain stable recordings from ciliary epithelial cells with such low resistance electrodes. Thus, the group of low potential values previously obtained and ascribed to the nonpigmented cells by the earlier authors were probably produced by im-

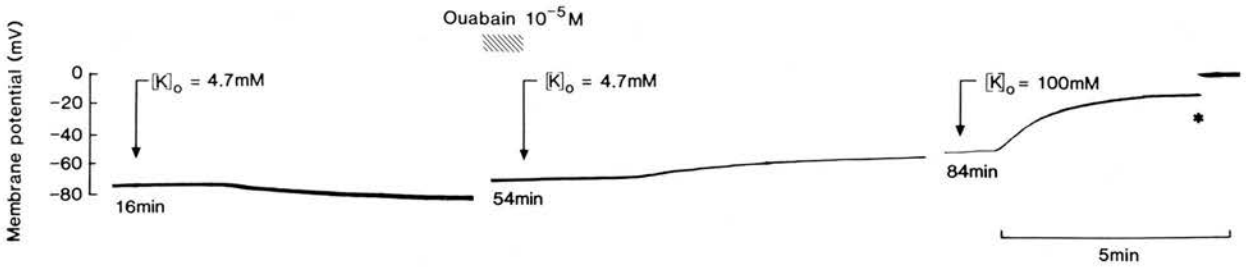

Fig. 9. Effect of ouabain on readmission responses to potassium from a ciliary epithelial cell. The recording on the left shows a response to potassium readmission before the application of ouabain at 52–54 min after cellular impalement. The cell was bathed in K^+-free Ringer from 5–16 min, 25–54 min, and 63–84 min after impalement (* indicates withdrawal of microelectrode).

palement damage. It appears that the membrane potential of both layers of ciliary epithelial cells is about −65 mV and conceivably this is an underestimate of the real value because of impalement artifact expected in recordings from small epithelial cells.[18] Dye-marking at the site of recording in this investigation has confirmed that the microelectrode tips were within ciliary epithelial cells. Difficulty was found, however, in discerning the precise location of the electrode tip during the majority of impalements. Thus, the mean membrane potential represents values from both cell layers. The cell-to-cell coupling, as evidenced by both dye transfer between cell layers (Fig. 2) and the electrotonic potentials, precludes against absolute definition of microelectrode tip location.

The evidence from experiments with double intracellular electrodes and dye ionophoresis indicates that the nonpigmented and pigmented cells are coupled in a functional syncytium. This finding agrees with electron microscopical studies that have revealed the presence of numerous gap junctions between ciliary epithelial cells.[22] Moreover, intracellular recording demonstrates that electrical coupling is effective over distances up to 300 μm (Fig. 3) and dye released into an impaled cell spreads through a very large group of neighbors (Fig. 2). Probably all of the ciliary epithelial cells are equipotential and function together in generating the transport of ions from the blood to the aqueous humor. In view of the small potential difference across the ciliary epithelium,[5,6,8,23] it is likely that the potential recorded across the basal membrane of the nonpigmented cell is almost balanced by a similar potential across the basal membrane of the pigmented cell.

Because the solution changes were made on the outer cell layer of the preparation and since the apical solution is probably unchanged by the experimental maneuvers, the effects of changes in the concentrations of the extracellular ions suggest that the basolateral membrane of the nonpigmented cells is permeable to potassium but less so to sodium, chloride or bicarbonate ions. Presumably the membrane potential should be close to the equilibrium potential for potassium (E_K) and more negative than the present mean estimate of the membrane potential, although lack of knowledge of the intracellular potassium concentration precludes a definitive statement on this point. If $[K]_i$ was 120 mM, which is a reasonable estimate for cellular [K], then $E_K \cong -82$ mV for $K_0 = 4.7$ mM. Removal of extracellular potassium caused a small hyperpolarization and not a profound depolarization as reported by Berggren.[9] The readmission of potassium produced a transient hyperpolarization even when $[K]_0$ was raised to 100 mM; (Fig. 7). A similar response to the readmission of 100 mM potassium has been observed in liver cells and the transient hyperpolarization attributed to an electrogenic efflux of sodium ions.[21] In such experiments, the local concentration of potassium at the surface of the epithelial cells during the hyperpolarization is not known because there were diffusion delays in the bathing solutions. Nevertheless, the local concentration was probably higher than the normal value of 4.7 mM during the hyperpolarization, and thus the membrane potential must have been more negative than E_K at $[K]_0 = 4.7$ mM.

Readmission responses were absent in ciliary epithelia bathed in ouabain-containing or Na-free solutions. Both conditions would prevent or reduce the active extrusion of sodium from the cells upon restoration of extracellular potassium. Similar evidence about potassium-evoked hyperpolarizations in conditions of sodium loading have been found in neurons,[24,25] smooth muscle,[24,26,27] liver cells,[21,28] pancreatic acinar cells,[29] and frog gastric mucosa[30,31] (the latter called an "anomalous" response to K^+) and taken as proof of the presence of an electrogenic Na/K pump. No doubt a similar electrogenic mechanism operates in ciliary epithelial cells to extrude sodium ions during sodium loading. Evidence has been obtained in the ciliary body that the Na:K pumps at the basolateral membranes of both cell layers are electrogenic[8] using 5×10^{-5} M ouabain, a concentra-

tion only slightly below that used here. In voltage-clamped neurons, Thomas[32] has shown that sodium injection evokes a transient outward current (peak value about 1 nA), which flows for about 10 min. The transient current was identified as an electrogenic sodium efflux. Since the surface area of these neurons is about 10^5 μm^2, the equivalent current density in a ciliary epithelial cell would produce a current of 10 pA. For a cell with an input resistance of 400 MΩ (see *Results*), this current would generate a hyperpolarization of 5 mV—a similar order to those reported here.

In ciliary processes bathed in Ringer solution, the electrogenic sodium pump apparently does not contribute to the membrane potential since ouabain did not cause a rapid depolarization upon application. Recently, Pesin and Candia[8] have presented evidence for Na/K pumps in the basolateral membranes of both the pigmented and nonpigmented cell on the basis of effects of ouabain and amphotericin B on the short-circuit current across the epithelium. Since the action of ouabain is rapid, the failure of ouabain to depolarize cells rapidly in our experiments strongly suggests that an electrogenic Na/K pump makes little or no contribution to the membrane potential under resting conditions (see below). However, ouabain produced a slow depolarization even after only a brief application. While 0.01 mM was ineffective in causing an alteration in resting membrane potential, this concentration was effective in eliminating the transient hyperpolarization caused by passing from a K^+-free solution to a K^+-containing solution (Fig. 9). Thus, while a ouabain concentration of 0.1 mM is needed to interfere with the pump under resting conditions, a concentration of 10^{-5} M (lower than that found to induce effects on the Na:K pumps in the whole ciliary body-iris preparation[8]) was effective in inducing changes in pump activity under conditions known to unmask the pump.[21,24-31] It appears, therefore, that ouabain binding is virtually irreversible and, in fact, cannot be reversed by a high concentration of extracellular potassium, which is known to interfere with ouabain binding in other cells.[33,34]

This initial study of the membrane potential of ciliary epithelial cells has indicated several interesting points, namely (1) the cell membrane is permeable to potassium; (2) the membrane has a low permeability to anions and sodium; (3) the cells have an electrogenic Na/K pump; and, finally, (4) all ciliary epithelial cells are electrically coupled. The contribution of these features to the role of the ciliary epithelium in the formation of aqueous humor remains to be examined. Thomas[35] has argued on the basis of the analysis given by Mullins and Noda[36] that the electrogenic component of the resting potential of cells in a steady state would be negligible if the sodium permeability P_{Na} were low relative to the potassium permeability P_K as found in ciliary epithelial cells. It seems likely that P_{Na}/P_K for the basal membrane of the nonpigmented cells is below 0.01 and thus an electrogenic Na/K pump might contribute only about 1 mV or less to the resting potential (see Fig. 2, Thomas[35]). In the nonsteady state, however, when cells are sodium-loaded, the enhanced pump activity could lead to a hyperpolarization as large as those reported here and elsewhere (see above).

Key words: rabbit, ciliary epithelium, intracellular potential, Na/K pump, ionic changes, cell-cell coupling

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CALCIUM-EVOKED OPENING OF POTASSIUM CHANNELS IN HAMSTER EGGS

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SUMMARY

Measurements of membrane potential and resistance have been made in zona-free hamster eggs. The resting potential lay in the range −9 to −100 mV and the input resistance fell in the range 14 to 440 MΩ; high resting potentials were associated with large input resistances. Calcium injected ionophoretically into an egg from an intracellular micro-electrode caused a reduction of the membrane resistance. The estimated reversal potential for the calcium-evoked response was about −80 mV and its amplitude depended on the extracellular concentration of potassium but not on the chloride concentration. We conclude that membrane potassium channels open in response to a rise in the cytosolic concentration of calcium ions. Evidence is presented to suggest that micro-electrode recordings of the membrane potential and resistance of eggs suffer from an impalement leak artifact. The presence of the artifact lowers the resting potential and resistance of the cell so that intracellular calcium injection causes a hyperpolarization. We conclude that a hyperpolarizing response to calcium would be unlikely in the absence of an impalement artifact.

INTRODUCTION

Miyazaki & Igusa (1981, 1982) have reported that the attachment of a hamster sperm to a hamster egg causes a series of transient hyperpolarizations of the egg's plasma membrane. It appears that calcium injection into a hamster egg mimics the effect of sperm attachment on the egg's membrane potential. However, Miyazaki & Igusa (1982) referred to unpublished results showing that calcium injection into hamster eggs produced a fall in membrane conductance whereas they presented evidence that sperm attachment evoked a transient increase in membrane conductance. It is difficult to reconcile these conflicting observations and therefore we decided to re-examine the effect of calcium injection on the membrane conductance of hamster eggs. A preliminary account of the work described in this paper has been given to the Physiological Society (Bland, Bountra, Georgiou & House, 1983).

METHODS

Egg donors

Mature virgin female golden hamsters, maintained under controlled lighting (8 h dark/16 h light), were injected I.P. with 30 i.u. pregnant mare serum gonadotrophin (Folligon; Intervet Labs. Ltd, Cambridge) in the early evening. Forty-eight hours later they were injected I.P. with 45 i.u. HCG (Chorionic Gonadotropin CG-2 Sigma Chemical Co., St Louis, U.S.A.).

The hamsters were killed, the oviducts opened and the eggs removed 15–18 h after the last injection.

Pre-treatment of eggs

All eggs were removed from the oviducts in a solution containing (mM): NaCl, 120; KCl, 5; $CaCl_2$, 4; $MgCl_2$, 1·2; Na lactate, 20; Na pyruvate, 1·0; glucose, 5·6; HEPES, 5; NaOH, 2·5. This medium, referred to as normal solution in this paper, had a pH of 7·3 and also contained bovine serum albumin

(4 mg/ml; Sigma). In some of the initial experiments the normal solution contained $CaCl_2$ at a concentration of 2 mM and was buffered at pH 7·6 with Tris–Tris Cl (Bland *et al.* 1983). The potassium concentration in the normal solution was varied by partial or complete replacement of NaCl by KCl. Similarly, the chloride concentration was changed by partial replacement of NaCl by Na propionate.

To remove the cumulus oophorus each egg was incubated for 2–4 min in normal solution containing Hyaluronidase (1 mg/ml; Type 1-S, Sigma). To remove the zona pellucida each egg, freed from cumulus, was bathed for 1–3 min in normal solution containing Trypsin (1 mg/ml; Type III, Sigma). The enzyme treatments were carried out at room temperature (20–22 °C).

Intracellular recording

An egg was placed in a chamber mounted on an inverted microscope (Biovert; Reichert, Austria). Normal solution at room temperature (20–22 °C) was pumped through the chamber (vol. 5 ml) by a Watson-Marlow H.R. Flow inducer (MRHE 200) at a rate of about 5 ml/min. The chamber contained two solid Ag/AgCl electrodes in contact with the solution.

Two micro-electrodes were inserted into the egg for potential recording and current passage. It was not necessary to immobilize the cell because it gently adhered to the glass base of the chamber. The first electrode (50 MΩ), filled with 2 M potassium acetate, was used for recording the intracellular potential relative to the first bath electrode. This micro-electrode was connected to the input of a high impedance pre-amplifier (Model KS 700; WP Instruments Inc., U.S.A.). Current pulses from a Devices stimulator triggered by a Digitimer (D4030; Devices Ltd) were passed between the barrel of the micro-electrode and the first bath electrode by means of a bridge circuit. The resulting electrotonic potentials were monitored for the determination of the current–voltage relation of the cell. The second micro-electrode (10 MΩ), filled with 1 M-$CaCl_2$, was used for the ionophoretic injection of calcium ions into the egg. Current pulses from a Devices stimulator were passed between one end of a 1 GΩ resistor in series with the barrel of the micro-electrode and the second bath electrode. The charge transfer through the calcium micro-electrode required to evoke the electrical responses described in this paper lay in the range 0·2–5 nC as observed previously by Miyazaki & Igusa (1982).

Permanent experimental records were obtained as pen recorder traces on a Devices M2 Recorder or as photographs of the screen of a storage oscilloscope (RM 5113, Tektronix Ltd).

RESULTS

Electrical properties of eggs

The resting potentials of eggs examined in this study lay in the range -9 to -100 mV, the mean $\pm$S.D. being -34 ± 16 mV for eighty-five cells. When a rectangular current pulse was passed through the recording micro-electrode an electrotonic potential was recorded. An example of the relation between applied current and voltage response is illustrated in Fig. 1. Evidently the relation between current and voltage is linear in the range 0 to -150 mV as found originally by Miyazaki & Igusa (1982) for hamster eggs. The input resistance was calculated from the linear part of the current–voltage relation; in the example shown in Fig. 1 the resistance was 170 MΩ. Mean $\pm$S.D. values for the input resistance of eighty-five cells were 147 ± 92 MΩ.

The electrical properties of nineteen eggs were examined in detail (Table 1) to give estimates of their time constants, resistances and capacitances. We measured the diameters of most of these cells so that their apparent surface areas could be estimated; in another set of measurements we found that the mean ($\pm$S.D.) diameter of fifty cells was 79 (± 3) μm. The specific membrane resistance is about 35,000 $\Omega \cdot cm^2$ and the membrane capacity is 3·0 $\mu F \cdot cm^{-2}$. It is possible that the resistance is underestimated and the capacitance over-estimated (see *Leak pathway*).

A hyperpolarizing current pulse evoked an anode-break response in most cells (Fig. 7*C*) provided that the membrane potential reached about -80 mV during the course of the pulse

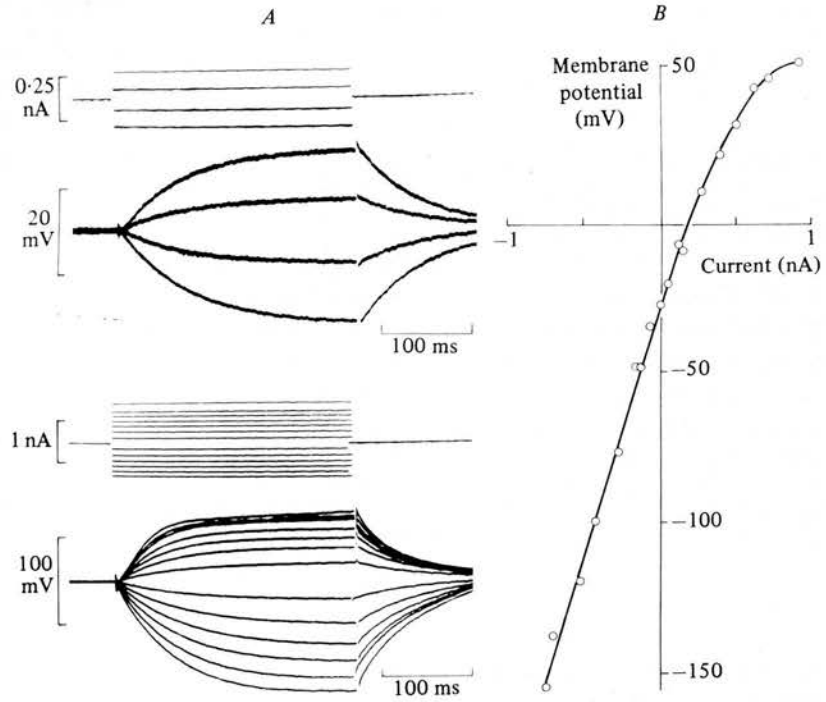

Fig. 1. Current–voltage relation of an egg. *A* shows current pulses passed through the intracellular micro-electrode and the corresponding electrotonic potentials recorded. *B* shows a plot of the relation between the applied current and the membrane potential of the egg.

Table 1. *Electrical properties of eggs*

Egg	Resting potential (mV)	Resistance (MΩ)	Time constant (ms)	Capacity (pF)	Membrane resistance kΩ . cm²	Membrane capacity μF . cm⁻²
1	−23	170	51	290	—	—
2	−44	360	240	660	—	—
3	−46	200	130	650	—	—
4	−48	180	130	720	—	—
5	−27	170	52	310	31	1·7
6	−31	250	63	250	46	1·4
7	−29	54	28	520	10	2·8
8	−28	100	48	460	—	—
9	−32	280	170	610	59	2·8
10	−29	110	76	690	24	3·2
11	−35	170	98	590	—	—
12	−43	130	93	700	—	—
13	−40	130	120	920	29	4·2
14	−28	96	88	920	18	4·9
15	−36	260	160	620	56	2·8
16	−29	100	70	690	—	—
17	−17	78	53	680	—	—
18	−36	180	100	570	35	2·9
19	−44	190	130	650	41	3·0
Mean ± S.D.	−34 ± 8	170 ± 78	100 ± 52	610 ± 180	35 ± 16 ($n = 10$)	3·0 ± 1·0 ($n = 10$)

as observed by Miyazaki & Igusa (1982). This phenomenon has been observed also in mouse eggs and is caused by the opening of voltage-sensitive calcium channels inactivated at the resting potentials usually observed (Okamoto, Takahashi & Yamashita, 1977).

Leak pathway

Continuous monitoring of resting potential and input resistance after an egg was impaled by a micro-electrode revealed that potential and resistance gradually increased over a period of about 10 min (Figs. 3–5). The progressive increases in potential and resistance suggest improved sealing of the micro-electrode to the cell membrane. Even when steady values of both potential and resistance have been reached it is possible that a significant leak impalement artifact remains (Hagiwara & Jaffe, 1979; Petersen, 1980). Miyazaki & Igusa (1982) reported that more negative resting potentials were associated usually with larger input resistances of hamster eggs but they provided no details. We have measured resting potential and input resistance for each cell, a suitable time being allowed for potential and resistance to attain steady values. There is a linear relation between them (Fig. 2). A simple equivalent circuit model with a variable leak resistance can describe the apparent relation in Fig. 2. If a leak pathway exists at the point of insertion of the micro-electrode then the effective input resistance, R', becomes $R_L R/(R_L+R)$ and the potential, E', is given by $(E_L R+ER_L)/(R_L+R)$ where R = cell resistance, R_L = leak pathway resistance, E = cell potential and E_L = leak pathway potential. It can be shown that R' and E' are linearly related by

$$E' = \left(\frac{E-E_L}{R}\right) R' + E_L$$

provided R, E and E_L are constant. According to this model the slope of the linear relation is $(E-E_L)/R$ and the intercept on the potential axis is E_L. Although it is not possible to vary R_L in order to test the hypothesis, the linear relation in Fig. 2 indirectly supports this model since it is expected that R_L will vary from cell to cell. It is also probable, of course, that R, E and E_L will vary from cell to cell so we decided to examine the relation between potential and resistance in individual eggs shortly after impalement. Fig. 3 shows the results of such an experiment. The upper part of the Figure is a pen trace of the membrane potential after micro-electrode insertion at zero time. The lower part is the corresponding graph of resistance and potential, the measurements being made at different times. Evidently there is again, in the case of a single cell, a linear relation between potential and resistance which is compatible with a progressive increase in R_L as the micro-electrode seals to the cell membrane.

A further kind of experimental result suggesting the presence of an impalement leak artifact is illustrated in Fig. 4. The crux of the experiment was to observe the effect of inserting a second micro-electrode (μe 2) on the membrane potential recorded by a previously inserted electrode (μe 1). The intracellular recordings show that the insertion of the second electrode caused a fall in the potential and resistance recorded by the first electrode. Both electrodes recorded the same membrane potential and electrotonic potentials generated by current pulses passed through the first electrode (Fig. 4).

Ionophoretic injection of calcium

We injected calcium ions ionophoretically into a hamster egg by passing a depolarizing current pulse through an intracellular micro-electrode filled with 1 M-$CaCl_2$ to see whether cytosolic calcium influences membrane permeability. The experimental protocol required

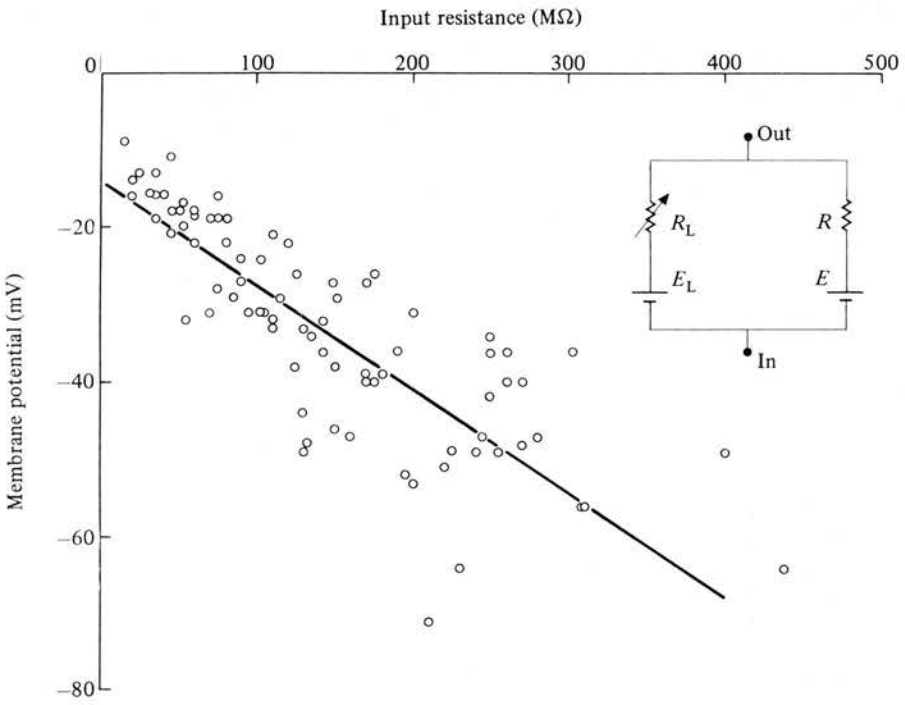

Fig. 2. Relation between estimates of input resistance and membrane potential of eggs. Each point indicates the values for a single egg. The line was obtained by linear regression analysis, the correlation coefficient being −0·7822 for 83 degrees of freedom ($P < 0{\cdot}001$). Inset Figure is an equivalent circuit model used to account for the relation between resistance and potential (see text). One egg had a resting potential of −100 mV and a resistance of 275 MΩ; this point has not been plotted on the graph although it has been included in the regression analysis.

the insertion of two micro-electrodes, the first for voltage recording and the second for calcium injection. Frequently the insertion of the calcium pipette was not satisfactory because it caused a permanent fall in resting potential and a drop in input resistance (Fig. 5*A*). However, occasionally the calcium pipette could be inserted successfully (Fig. 5*B*). A satisfactory insertion was indicated by a transient hyperpolarization and a transient fall in resistance. Even after a satisfactory impalement by the calcium pipette success was not assured because sometimes the pipette failed to pass sufficient current or developed a poor current-passing property during the experiment.

In forty-five successful experiments a depolarizing current pulse through the calcium pipette was followed by a hyperpolarization lasting 10–30 s. The amplitude and duration of the hyperpolarization evoked by ionophoretic injection of calcium were graded with the quantity of charge ejected from the calcium pipette (Fig. 6). Responses to large ionophoretic pulses of calcium were prolonged and their amplitudes were reduced by raising the concentration of potassium in the bathing fluid (Fig. 6).

In control experiments where the calcium pipette was replaced by a similar one filled with 2 M potassium acetate it was found that depolarizing currents did not elicit hyperpolarizing responses (Fig. 7*A*). In some cases (not illustrated) a small depolarizing response followed the pulse possibly because of temporary resistive breakdown of the membrane.

By contrast, a hyperpolarizing current pulse through the control pipette or a calcium pipette occasionally produced a small transient hyperpolarization (Fig. 7*A*, *B*).

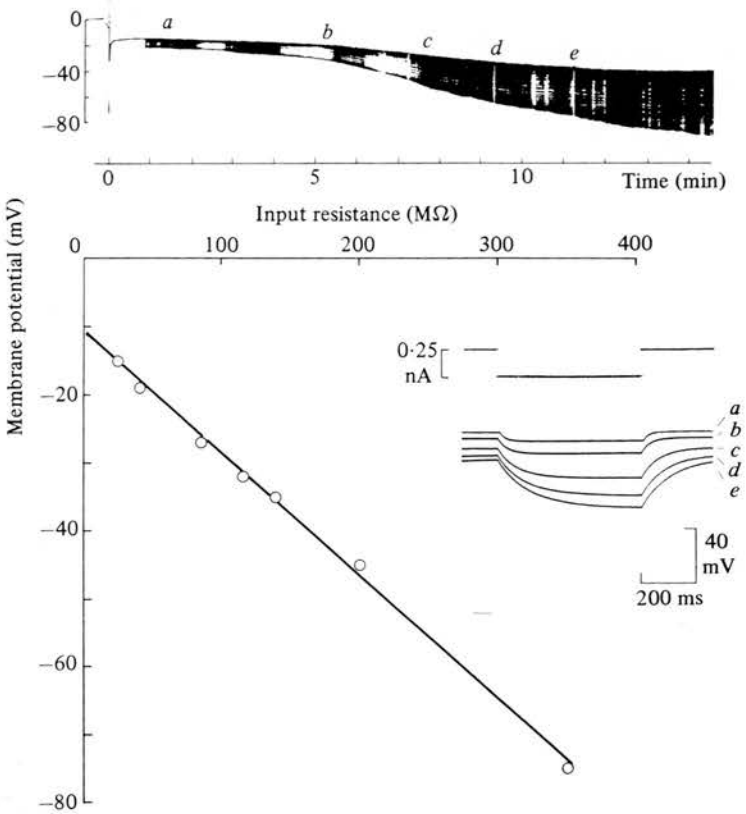

Fig. 3. Progressive increase in the membrane potential and the input resistance of an egg after impalement by a micro-electrode. The upper part is a pen tracing showing the initial period of recording after impalement, the downward deflexions being produced by constant current pulses passed through the recording micro-electrode. Below is a plot of the membrane potential against the input resistance measured at times (*a*–*e*) during the recording. The potential and resistance continued to increase (not shown) to maximum values of −75 mV and 350 MΩ respectively. Oscilloscope pictures of current pulses and electrotonic potentials recorded at *a*, *b*, *c*, *d* and *e* are superimposed beside the graph. The line was obtained by linear regression analysis, the correlation coefficient being −0·9990 for 5 degrees of freedom ($P < 0{\cdot}001$).

The small responses to hyperpolarizing current pulses passed through the calcium or control pipette probably are caused by the calcium influx responsible for the anode-break response recorded in hamster eggs (Fig. 7*C*) (Miyazaki & Igusa, 1982). We have found that anode-break responses are blocked by the presence of cobalt or lanthanum ions in the bathing solution.

To monitor the change in membrane conductance produced by calcium injection we passed constant current pulses through the recording micro-electrode. Because the current–voltage relation of the hamster egg is linear in the range 0 to −150 mV hyperpolarizing current pulses were applied. Calcium injection caused a rise in membrane conductance as judged by the reduction of the electrotonic potentials which are proportional to membrane resistance (Fig. 8). Generally the fall in resistance outlasted the hyperpolarization.

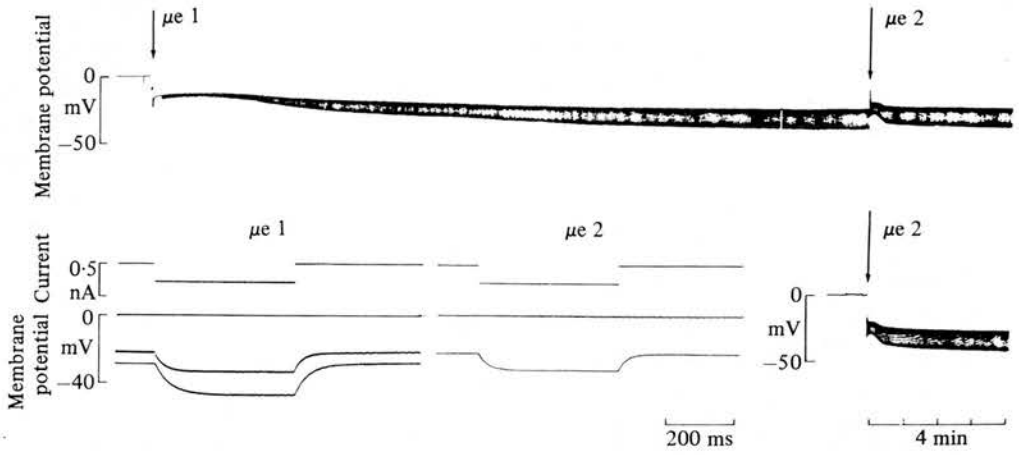

Fig. 4. Insertion of two micro-electrodes into a hamster egg. The upper pen trace shows the membrane potential recorded by the first micro-electrode (μe 1) before and after insertion of the second micro-electrode (μe 2). The downward deflexions were produced by constant current pulses passed through the first micro-electrode. The lower pen trace shows the membrane potential recorded by the second micro-electrode, the downward deflexions being caused by the current pulses passed through the first micro-electrode. Alongside the lower pen trace are oscilloscope pictures of current pulses and electrotonic potentials recorded by both micro-electrodes just after insertion of the second micro-electrode. The lower oscilloscope trace recorded by the first micro-electrode shows the electrotonic potential recorded just before the insertion of the second micro-electrode.

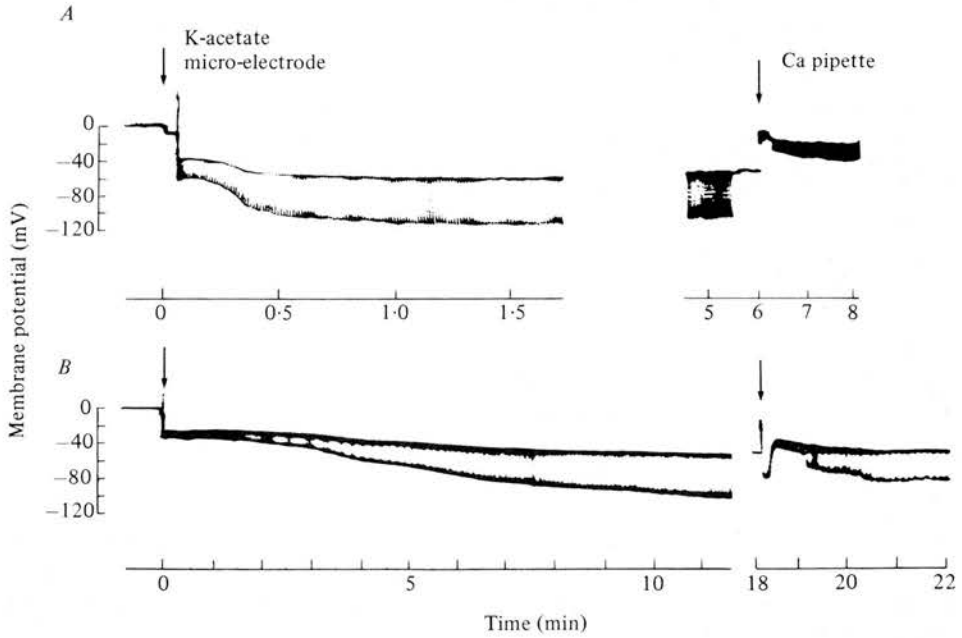

Fig. 5. Insertion of two micro-electrodes into an egg, one being filled with potassium acetate and the other with calcium chloride. The downward deflexions were produced by constant current pulses passed through the potassium acetate micro-electrode and the arrows indicate the times at which attempts were made to insert the electrodes. *A* shows the trace of the membrane potential recorded by the potassium acetate electrode. At the second arrow the attempt to insert the calcium pipette failed since there was a fall in both membrane potential and resistance. *B* shows the results of another experiment in which the attempt to insert the calcium pipette (second arrow) was successful since the changes in potential and resistance were transient only (full recovery not shown).

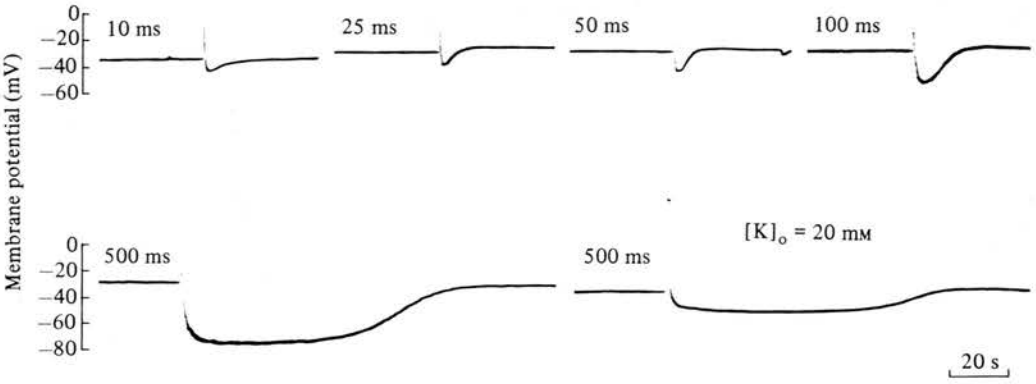

Fig. 6. Effect of the duration of the current pulse passed through a calcium pipette on the membrane potential of an egg. The pulse durations are given above each section of the trace showing responses to injected calcium. A maximum response was obtained with a pulse duration of 500 ms and this response was reduced when the extracellular concentration of potassium was increased from 5 to 20 mM.

Analysis of records

As indicated by Trautwein & Dudel (1958), values for the conductance, g and the reversal potential, e, at the peak of a response may be derived from a single record of the kind shown in Fig. 8. Relations equivalent to those of Trautwein and Dudel are derived here as follows. Evidently for a current pulse I giving electrotonic potentials P and p at rest and during the response respectively we have

$$P = \frac{I}{G} \quad \text{and} \quad p = \frac{I}{G+g},$$

where G is the resting conductance. For a resting potential E and a response v we find

$$g = \left(\frac{P-p}{p}\right) G$$

and

$$e = \left(\frac{P}{P-p}\right) v + E.$$

These relations would not be valid unless the resting conductance were independent of the current and hence of the membrane potential as we have found (Fig. 1).

To illustrate the method of analysis we consider the examples shown in Fig. 8. The intracellular recording in Fig. 8 (upper) shows $E = -19$ mV, $v = -30$ mV, $P = -20$ mV and $p = -10$ mV and these yield $e = -79$ mV. The corresponding values in Fig. 8 (middle) are $E = -35$ mV, $v = -29$ mV, $P = -87$ mV and $p = -42$ mV and hence $e = -91$ mV. By this method we determined the reversal potentials for calcium-evoked responses in thirty-three cells (Table 2).

During the course of experiments we noticed that spontaneous hyperpolarizations occurred in some eggs. Fig. 9 shows two striking examples of large spontaneous hyperpolarizations. Applying the analysis outlined above we have estimated the reversal potentials for these spontaneous events to be -80 and -90 mV. These values fall within the range observed in the calcium injection experiments. The mean value of the reversal potential for

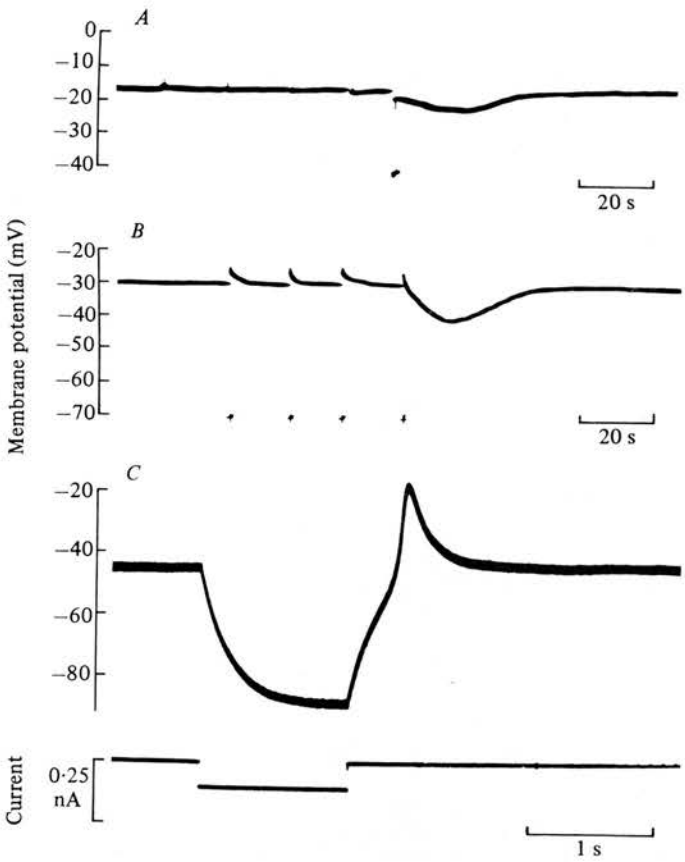

Fig. 7. Effect of current passage through micro-electrodes on the membrane potential of eggs. *A* shows the effect of large current pulses passed through a low resistance micro-electrode filled with 2 M potassium acetate. The first four pulses were depolarizing and had durations of 100, 250, 500 and 1000 ms respectively; the fifth pulse was equal but of opposite polarity to the preceding pulse. *B* shows the effect of large hyperpolarizing current pulses passed through a calcium pipette on the membrane potential recorded by a potassium acetate micro-electrode inserted into the same cell. *C* shows the effect of a hyperpolarizing current pulse (lower trace) passed through a potassium acetate micro-electrode on the membrane potential (upper trace) recorded by the same electrode. Note the anode-break response at the end of the hyperpolarizing electrotonic potential.

the response to calcium is −79 mV and this suggests that calcium causes an increase in membrane permeability to chloride or potassium ions.

Ionic basis of response to calcium

We injected calcium into four eggs bathed in a solution containing a chloride concentration of 10 mM. This solution caused depolarization of each egg and the mean resting potential fell from −38 to −24 mV. Calcium injection caused a hyperpolarizing response with a mean reversal potential of −74 mV. Thus the potential change produced by a rise in cytosolic calcium concentration does not depend on the opening of chloride channels.

The resting potential of hamster eggs is relatively insensitive to the potassium concentration in the bathing solution (Miyazaki & Igusa, 1982) as we have confirmed in this study. In

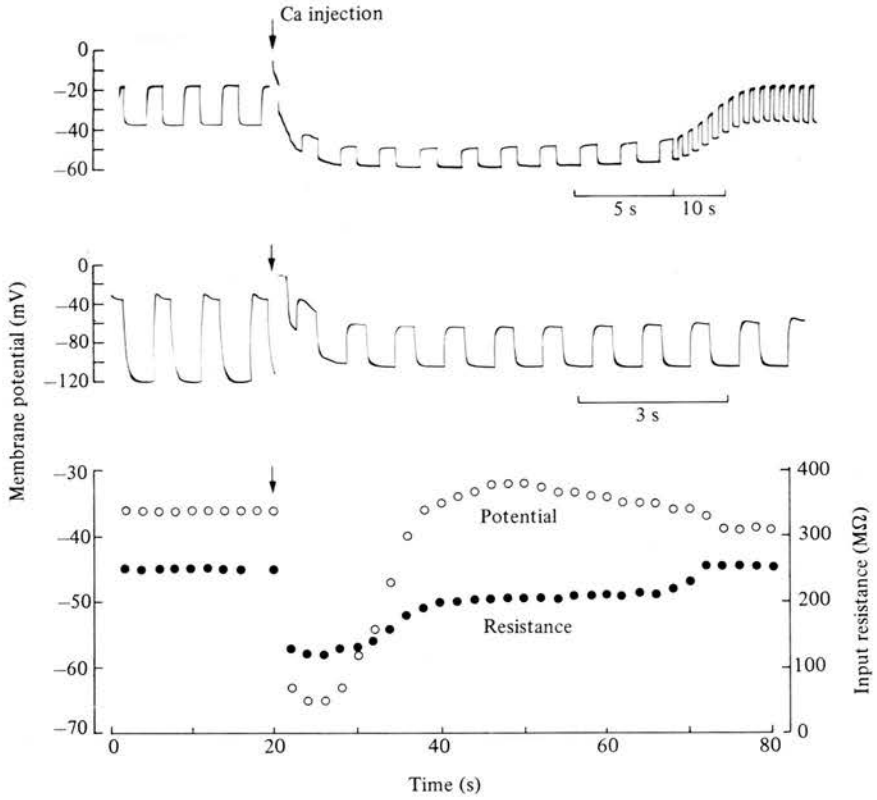

Fig. 8. Effect of calcium ionophoretically injected into eggs on their membrane potential and resistance. Arrows indicate the time at which calcium was injected ionophoretically into the eggs. Upper and middle traces show results of two experiments on different eggs. The hyperpolarizing electrotonic potentials were produced by constant current pulses passed through the recording micro-electrodes. The graph displays the results of the entire response to calcium which is illustrated partially by the middle trace.

Table 2. *Reversal potential* for response to injected calcium*

Resting potential (mV)	Input resistance (MΩ)	Reversal potential (mV)	Response to calcium	
			Hyperpolarization (mV)	Resistance† (MΩ)
-37 ± 14	160 ± 73	-79 ± 13	23 ± 9	50 ± 30

* Mean ± S.D. values (thirty-three cells).
† Minimum value.

each of eight eggs it was possible to determine the reversal potential for the response to calcium injection at two different concentrations of potassium. An example is illustrated in Fig. 10 which shows the electrotonic potentials produced by constant current pulses under resting conditions and during responses to calcium when the cell was bathed in solutions containing 1 or 125 mM potassium. The reversal potential when $[K]_o = 1$ mM was -98 mV and when $[K]_o$ was changed to 125 mM it became -13 mV. The shift of 85 mV is less than

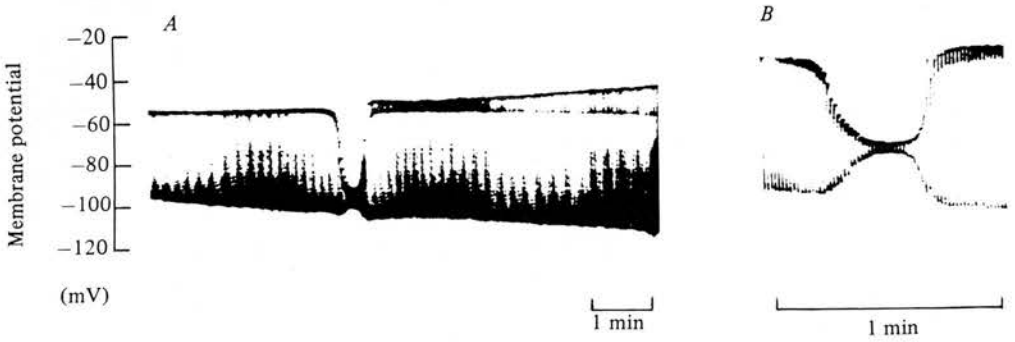

Fig. 9. Examples of spontaneous hyperpolarizations recorded from eggs. The brief downward deflexions in *A* and *B* were produced by constant current pulses passed through the recording micro-electrode.

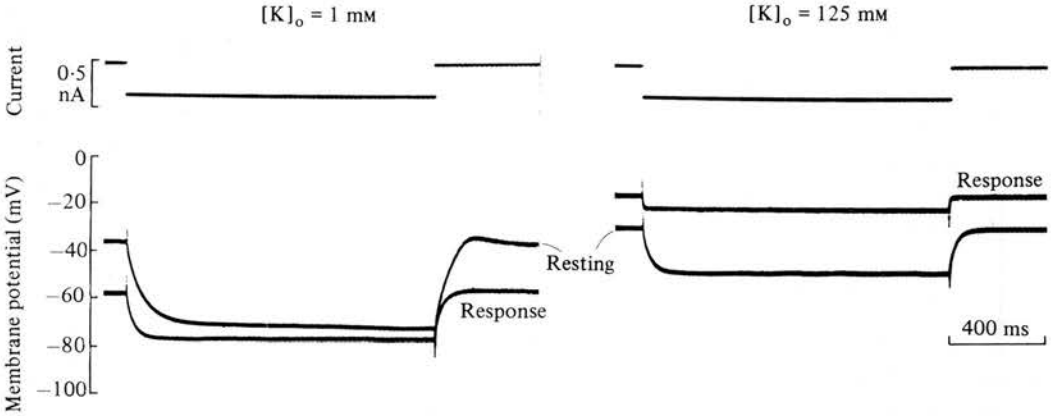

Fig. 10. Effect of the extracellular concentration on the electrical response to calcium injection into an egg. Upper and lower traces are superimposed traces of current pulses and electrotonic potentials respectively. When $[K]_o$ = 1 mM the response to calcium was a hyperpolarization whereas when $[K]_o$ = 125 mM the response was a depolarization. In both conditions the amplitudes of the electrotonic potentials were reduced.

the theoretical value of 122 mV according to Nernst. In contrast, the corresponding shift of the resting potential was from −42 to −33 mV. The mean slope of the relation between the reversal potential and log $[K]_o$ for the results shown in Fig. 11 is 50 mV whereas that expected according to Nernst is 58 mV.

Values of the reversal potential have been determined at external concentrations of potassium of 1, 5, 40 and 125 mM (Fig. 11). The strong dependence of the reversal potential on potassium concentration indicates that calcium injection opens potassium channels in the plasma membrane of hamster eggs.

An indirect finding which strongly supports the idea that cytosolic calcium controls the potassium permeability of hamster eggs is shown in Fig. 12. In this experimental record, which is representative of several others, a single intracellular electrode monitored changes in the resting potential which occurred when hyperpolarizing current pulses were passed through it. Trains of hyperpolarizing current pulses caused hyperpolarizing responses when $[K]_o$ = 1 or 5 mM and a depolarizing response when $[K]_o$ = 125 mM. We interpret this record to mean that the anode-break responses evoked by hyperpolarizing pulses produce a sufficient rise in cytosolic calcium concentration to open membrane potassium channels.

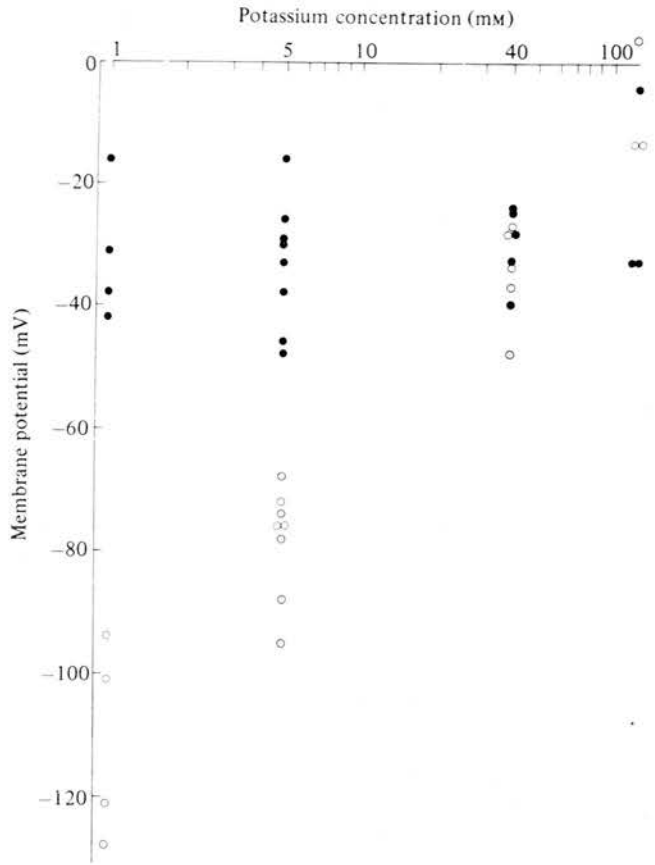

Fig. 11. Effect of the extracellular concentration of potassium on the resting potential (●) and the estimated reversal potential (○) in the same cell during the response to injected calcium.

In separate experiments we found that the hyperpolarizing response of an egg to a hyperpolarizing current pulse passed through the calcium pipette was reversibly abolished by the presence of 1 mM lanthanum nitrate in the bathing solution whereas the hyperpolarizing response to injected calcium was not abolished by lanthanum ions.

DISCUSSION

The main object of our experiments was to establish whether or not ionophoretic injection of calcium ions into hamster eggs altered plasma membrane resistance.

We have shown that plasma membrane potassium channels in the hamster egg are opened directly by ionophoretic calcium injection or indirectly by calcium influx by electrical excitation of the membrane. A rise in cytosolic calcium concentration produces an observable increase in membrane conductance which underlies a hyperpolarizing response with a potassium-dependent reversal potential as found for the sperm-evoked response in hamster eggs by Miyazaki & Igusa (1982). Such a calcium-mediated increase in membrane potassium permeability has been found in numerous cells including erythrocytes (Gardos, 1958), neurones (Meech, 1978) and gland cells (Petersen, 1980).

In common with previous findings the time course of the calcium-evoked electrical

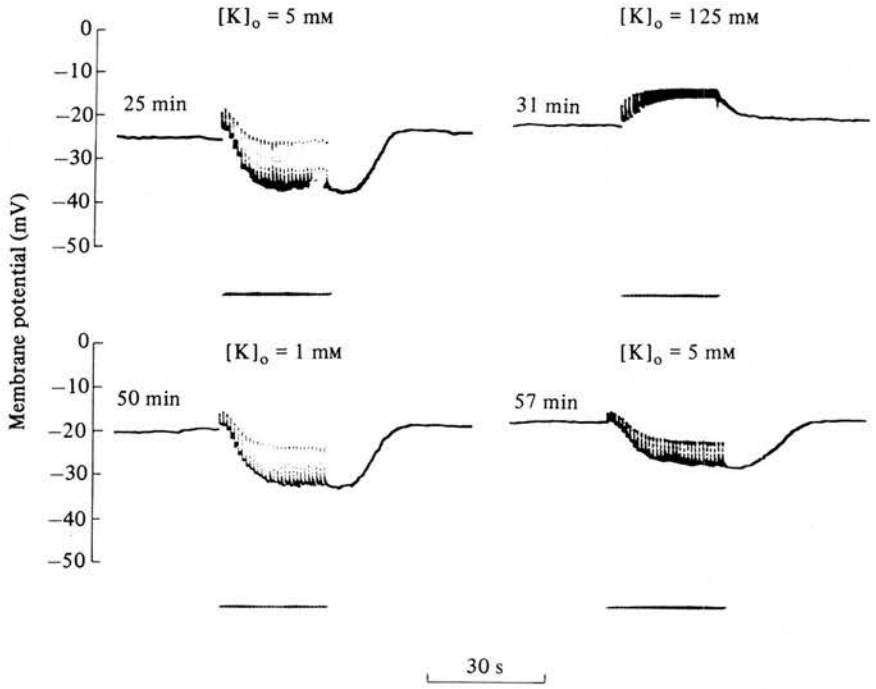

Fig. 12. Responses of an egg bathed in different potassium concentrations to trains of hyperpolarizing current pulses passed through the recording micro-electrode. The times after impalement at which the current pulses were delivered, are given above each extract from the continuous record of the membrane potential. The horizontal bars underneath each trace were generated by the pen hitting the physical end of its travel in the recorder.

response is relatively slow in hamster eggs. For example, the time-to-peak, t_p, is about 3 s. On the assumption that the tip of the calcium pipette acts as a point source during the ionophoretic pulse then $t_p = x^2/6D$ where x is the diffusion pathlength between the pipette tip and the membrane and D is the effective diffusion coefficient (see del Castillo & Katz, 1955). The value of x must be less than the cell's radius, i.e. 40 μm. Hence on a diffusion model of the response to calcium D must be smaller than 10^{-6} cm^2 . s^{-1} which is plausible in view of its slow diffusion in aqueous solution and of the calcium buffering power of cytoplasm (cf. Hodgkin & Keynes, 1957).

Although the results presented in this paper suggest that calcium injection can mimic the electrical effect of sperm attachment to hamster eggs it is not known how sperm attachment might produce the necessary increase in cytosolic calcium concentration (Miyazaki & Igusa, 1982).

Our electrical measurements with intracellular micro-electrodes point to the existence of a leak pathway at the electrode tip. This leak pathway is sufficient to cause depolarization of the egg. It is our view that previously published values of the potential and resistance of mammalian eggs (Powers & Tupper, 1974; Okamoto *et al.* 1977; Miyazaki & Igusa, 1982) are probably underestimates of the real values. In conditions where the hamster egg is depolarized by the leak impalement artifact the calcium-evoked opening of potassium channels produces a hyperpolarization. It is possible that the real potential of the egg is close to or even exceeds the equilibrium potential for potassium and therefore probable that potassium channel opening causes no change of potential or depolarization.

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Note added in proof. While this paper was in press Igusa & Miyazaki (1983) reported that calcium injection into hamster eggs causes a rise in membrane conductance as described here.

In recent experiments we have found that hamster and mouse eggs with high membrane potentials (−90 mV) and resistances (500 MΩ) show overshooting action potentials in response to depolarizing current pulses.

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Calcium injection into hamster eggs causes hyperpolarization and decrease in membrane resistance

By K. P. Bland, C. Bountra, P. Georgiou and C. R. House. *Department of Veterinary Physiology, University of Edinburgh, Edinburgh EH9 1QH*

When a sperm attaches to a hamster egg the egg's plasma membrane undergoes a transient hyperpolarization and a fall in resistance (Miyazaki & Igusa, 1982). Moreover, ionophoretic injection of calcium ions into hamster eggs evokes a similar hyperpolarization although there has been no direct test that calcium also produces a reduction in membrane resistance. We report here that calcium injection into hamster eggs causes a large decrease in membrane resistance.

Eggs were obtained by superovulation from mature female golden hamsters. After removal from the oviducts the eggs were bathed at room temperature (20–22 °C) in a solution containing (mM): NaCl, 120; KCl, 5; $CaCl_2$, 2; $MgCl_2$, 1·2; glucose, 5·6; Na lactate, 20; Na pyruvate, 1; Tris-Tris Cl pH 7·6, buffer 5. Bovine serum albumin (4 mg/ml) was added to the solution. Cumulus was removed by bathing eggs in solution containing Hyaluronidase (1 mg/ml) for 2–4 min and then the zona pellucida was removed by transfer into a solution containing Trypsin (1 mg/ml) for 1–3 min.

A micro-electrode (50 MΩ) filled with 2 M-K acetate was inserted into an egg for potential recording; current pulses were passed through this intracellular electrode by a bridge circuit for input resistance measurement. Calcium was injected ionophoretically into the egg by passing current pulses (0·2–5 nC) through a second intracellular micro-electrode (10 MΩ) filled with 1 M-$CaCl_2$.

Intracellular recordings have been made in twenty hamster eggs. The mean ± S.D. values for resting potential and input resistance were -32 ± 14 mV and 135 ± 55 MΩ respectively. Upon calcium injection each egg underwent a transient hyperpolarization lasting about 30 s and a transient fall in its input resistance which usually outlasted the potential change. The mean values (± S.D.) for the minimum membrane potential and input resistance during the response to calcium were -53 (± 13) mV and 44 (± 29) MΩ. Thus intracellular calcium injection produces a large fall in membrane resistance. Analysis of the electrical response according to a simple equivalent circuit model (Trautwein & Dudel, 1958) yields a reversal potential of -73 ± 13 mV (mean ± S.D.).

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Action potential in unfertilized mouse egg

By K. P. Bland, C. Bountra, P. Georgiou and C. R. House. *Department of Veterinary Physiology, University of Edinburgh, Edinburgh EH9 1QH*

Previous work on unfertilized mouse eggs (Powers & Tupper, 1974; Okamoto, Takahashi & Yamashita, 1977) has yielded low resting potentials (−14 to −45 mV), input resistances in the range 20–200 MΩ and anode-break responses to hyperpolarizing current pulses. However, these intracellular recordings probably suffered from impalement leak artefacts, because we have found that after allowing a suitable period for sealing of the micro electrode to the cell membrane, large membrane potentials of −80 mV and input resistances of about 500 MΩ are observed. In this condition action potentials occurred in response to brief depolarizations.

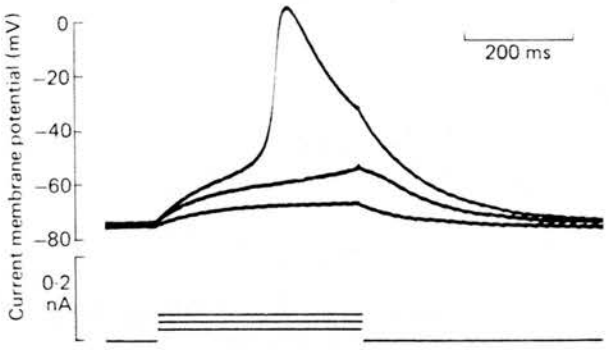

Fig. 1. Superimposed intracellular recordings from an egg. Upper traces are responses to current pulses (lower traces) passed through the recording micro-electrode (50 MΩ) filled with 2 M-K acetate. The egg was bathed at 24 °C in a solution (pH 7·3) containing (mM): choline Cl, 120; KCl, 5; $CaCl_2$, 4; $MgCl_2$, 1·2; glucose, 5·6; HEPES, 5; NaOH, 2·5. Methods of obtaining zona-free eggs from mice have been described by House & Bland (1983).

Action potentials have been recorded from zona free eggs (Fig. 1) and also from eggs with zona pellucida. The peak value of the action potential depended on the external concentration of calcium but was independent of sodium concentration. Cobalt chloride (5 mM) in the bathing solution caused an almost complete block of the action potential and this block was reversible. We conclude that the mouse egg has a calcium-dependent action potential.

Supported by the Wellcome Trust and Faculty of Veterinary Medicine, Edinburgh University. P. Georgiou is a Harriet Thomson Scholar and C. Bountra is an M.R.C. scholar.

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